An unusual postharvest spotting disease of the commercial mushroom, *Agaricus bisporus*, caused by a novel pathovar of *Pseudomonas tolaasii*

by

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Abstract

An unusual postharvest spotting disease of the commercial mushroom, *Agaricus bisporus,* which was observed on a commercial mushroom farm in Ontario, was found to be caused by a novel pathovar of *Pseudomonas tolaasii.*

Isolations from the discoloured lesions, on the mushroom pilei, revealed the presence of several different bacterial and fungal genera. The most frequently isolated genus being *Pseudomonas* bacteria. The most frequently isolated fungal genus was *Penicillium.* Of the bacteria and fungi assayed for pathogenicity to mushrooms, only *Pseudomonas tolaasii* was able to reproduce the postharvest spotting symptom. This symptom was typically reproduced 1 to 7 days postharvest, when mushroom pilei were inoculated with 10^1 to 10^5 cfu. Of the fungi tested for pathogenicity only a *Penicillium* sp. and *Verticillium fungicola* were shown to be pathogenic, however, neither produced the postharvest spotting symptom.

The *Pseudomonas tolaasii* strain isolated from the postharvest lesions differed from a type culture (*Pseudomonas tolaasii* ATCC 33618) in the symptoms it produced on *Agaricus bisporus* pilei under the same conditions and at the same inoculum concentration. It was therefore designated a pathovar. This strain also differed from the type culture in its cellular protein profile. Neither the type culture, nor the mushroom pathogen was found to contain plasmid DNA. The presence of plasmid DNA is therefore not responsible for the difference in pathogenicity between the two strains.

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Introduction

A postharvest disease of the commercial mushroom, *Agaricus bisporus* (Lange) Imbach, was observed on a commercial mushroom farm in Ontario. The unusual lesions produced on these mushrooms appeared several days after harvest and were characterized as being chocolate brown in colour with a lighter purple brown to reddish brown periphery. These spots were usually less than 4mm in diameter and numbered up to ten per mushroom cap. Older lesions tended to be darker in colour and were sunken. The mushrooms were of otherwise good quality and colour (figure 1). This postharvest problem has affected up to 5% of the total mushrooms produced at this farm during the most severe outbreak. This is quite substantial since this farm produces over 180 000 kg of mushrooms per week.

Agaricus bisporus is the most extensively cultivated mushroom in Canada. Its production exceeds 73 million kg per year with approximately 73% of these mushrooms sold on the fresh market (Potter, 1999). General consumer preferences require a high quality *A. bisporus* to be completely white, with a closed veil, short stipe and no visible gills (Ryall and Lipton, 1979). It should also have a tender texture and the characteristic mushroom odour (Burton and Noble, 1993). In addition, mushrooms should be free of any discolourations due to bruising or microbial contamination, which decrease postharvest quality and therefore consumer appeal in the marketplace.

Spotting of *Agaricus bisporus* by bacterial or fungal pathogens is quite common in the mushroom industry. However, the spotting symptoms are usually observed prior to

Figure 1. Postharvest spotting disease of the commercial mushroom, *Agaricus bisporus*. This is a sample of mushrooms which were returned from retail after the mushrooms developed the postharvest spotting symptom.

harvest. Growers can usually bring these problems under control by managing the environment, personnel or through the use of pesticides.

Based on the problem, the objectives of this research were: (1) to identify the organism(s) responsible and (2) to bring this postharvest problem under control, through, for example, the use of pesticides or environmental manipulation. The second objective was beyond the scope of this thesis, however, and was therefore delegated to future research.

Literature Review

Agaricus bisporus (commercial mushroom)

Agaricus bisporus belongs to the Division Basidiomycota in the Kingdom Fungi. All members of this division share the formation of basidiospores externally on a basidium (Moore-Landecker, 1996). *Agaricus bisporus* belongs to the Class Basidiomycetes. Members of this class are characterized by the forms of their basidia and by the structures supporting the basidia (Griffin, 1994). The Class Basidiomycetes is subdivided into 17 orders, one of which is the Order Agaricales to which *A. bisporus* belongs (Moore-Landecker, 1996). The agarics have gills over the surface of which the basidia are formed in an extensive hymenium (Moore-Landecker, 1996). Agarics are universally distributed and grow in a variety of habitats, including soil, wood and dung. The genus *Agaricus* is a large one and almost all species are edible. *Agaricus bisporus* gets its species name because it only produces two spores on each basidium (Moore-Landecker, 1996).

Mushroom cultivation

The Ontario mushroom industry is flourishing. Commercial mushrooms are the second most valuable table crop in Ontario (Rinker, personal communication). In 1998 the consumption of mushrooms was in excess of 78 million kg (Potter, 1999). In 1998, over 38 million kg of mushrooms were produced in Ontario with a farm-gate value of over $117 million dollars for growers. Farm gate price for fresh, high quality mushrooms

in 1998 was $3.10/kg (Potter, 1999). Ontario mushroom farmers returned approximately $47 million to the Ontario economy in 1998 through wages and salaries (Potter, 1999).

Methods of Cultivation

Composting

Commercial mushrooms are produced on a specially prepared material called compost. Mushrooms are saprophytic organisms and rely on non-living organic matter for their nitrogen and carbon. The primary function of the composting process is to prepare a uniform material which is highly selective for growth and production of the commercial mushroom. Straw-bedded horse manure is the basic ingredient for the preparation of compost (Rinker, 1993). It contains a high percentage of straw (carbon source), ammonia (nitrogen source) and is a source of several microorganisms which aid in the composting process (Rinker, 1993). During composting the only component not being broken down by the compost microflora is lignin (Smith and Aidoo, 1988). The left over lignin cannot be broken down by the compost microbes and lignocellulose then serves as the main selective nutrient for *A. bisporus*. Composting then provides the *A. bisporus* mycelium with insoluble substances which it is capable of degrading for nutrition, but which other microorganisms cannot use as nutrition, so that their growth is inhibited.

There are two composting phases. The purpose of Phase I composting is to mix and wet the straw-bedded horse manure and begin the composting process (Ingold and Hudson, 1993). Phase I requires approximately 7 to 21 days. The raw material is formed

into large heaps and is turned and wetted before being shaped into the long narrow stacks to continue the composting process called ricks (Rinker, 1993). The center of the compost stack may reach 85°C, which is high enough to kill most of the pests and pathogens which may be present. However, the outer layers never reach this temperature and as a result are susceptible to contamination (Fletcher *et al.,* 1989). Phase I is complete when the material becomes pliable, is a dark brown colour and has a sharp ammonia odor (Rinker, 1993).

Phase II, also referred to as peak-heating, pasteurization or cook-out, allows the fermentation process to continue until the compost is both suitable and selective, nutritionally, for the growth of mushroom mycelium and also eliminates pests and pathogens which are harmful to *A. bisporus* (Fletcher *et al.*, 1989). Careful control of temperature, relative humidity and oxygen levels are critical to the cookout process. Compost should be pasteurized at a maximum of 60°C and under high relative humidity conditions for 6-8 hours (Ingold and Hudson, 1993). Following pasteurization the compost must be conditioned. Temperatures are gradually reduced to 45-50°C and fresh air must be supplied to maintain aerobic conditions. (Fletcher *et al.*, 1989). The ammonia smell will gradually disappear, indicating the completion of the conditioning period (Rinker, 1993). After an additional cool-down period, the compost will be ready for spawning.

Spawning

The propagating material used in mushroom cultivation is called spawn. Spawn consists of sterilized grains that have been thoroughly colonized by mushroom mycelium (Deacon, 1984). The grains may be wheat, millet or rye (Fletcher *et al.,* 1989). Spawning is the process of mixing the spawn into the compost. Spawn is usually applied at a rate of 0.5 kg/m^2 or 0.5% on a wet weight basis (Rinker, 1993). A compost temperature of 24-26°C with 90-95% relative humidity and a carbon dioxide level of 1-2% is ideal for spawn run (Fletcher *et al.,* 1989). Spawn running is colonization of the compost from the grain inoculum. It is generally accepted that modern spawn is free from pests and pathogens due to the strict hygiene measures practiced during manufacture (Rinker, 1993). At spawning, however, it becomes vulnerable to contamination. Introduction of disease during spawn run can be very damaging due to early stage of production. Spawn run takes approximately 14 days, at which time 80-90% of the compost surface will show a white coating of mycelium (Rinker, 1993). At the completion of spawn run a casing material can then be applied.

Casing

To promote sporophore production by *Agaricus bisporus*, a relatively biologically inert material is added as a surface layer to the spawn run compost. This casing layer is usually a mixture of sphagnum peat moss and calcitic limestone ($CaCO_3$) in a ratio of 1:1

on a dry weight basis. The $CaCO_3$ acts as a neutralizing agent to raise the pH of the peat moss from 3.5-4.5 to the desired pH of 7.5 (Rinker, 1993). The casing is applied 5-8 cm deep. The casing layer provides stimulation of fruiting and anchorage for the sporophores. In addition, it has good water-holding reserves which are essential for high yields (Fletcher *et al.,* 1989). Casing material is easily contaminated and if this occurs can result in serious outbreaks of pests and diseases. Casing material can be treated with heat or chemicals to remove any undesirable organisms. However, most growers do not pasteurize their peat moss since oversteaming could destroy bacteria which are important in mushroom formation, substances which are toxic to mushroom growth may be released and breakdown of the peat itself could occur (Rinker, 1993). Strict sanitation measures should therefore be employed to decrease the possibility of contamination.

The temperature in the compost should be kept at the same level as during spawn run (24-26°C) for the first week following casing (Rinker, 1993). Carbon dioxide levels, greater than 0.1%, with high relative humidity, between 90-95%, will stimulate the vegetative stage of the mushrooms (Rinker, 1993). Once the mycelium has reached the surface of the casing the mushroom is induced to form primordia by reducing both the air temperature to 16-18°C and the CO_2 concentration in the air to 600-1000 ppm (Fletcher *et al.,* 1989).

Cropping

Mushrooms develop in well defined cycles, termed "flushes" or "breaks", the first break beginning approximately three weeks after casing and continuing at roughly weekly intervals (Fletcher *et al.*, 1989). In commercial practice three breaks are usually

picked before the crop is removed to make room for the next crop (Rinker, 1993). Individual breaks tend to produce progressively fewer mushrooms, the greatest yield of mushrooms normally being picked on the first flush. Mushrooms are harvested when the cap is at its maximum size and before the veil under the cap stretches and opens. Mushroom yields vary considerably depending on growth conditions. The Ontario average yield was 2.0 kg/sq.ft. per crop in 1998 (Potter, 1999). The casing is watered at regular intervals after its application and pickers move in and out of the growing rooms to harvest crops. Any pests or diseases, which do occur usually, increase in incidence during the life of the crop (Fletcher *et al.,* 1989). Not only do these pests and diseases spread within the crop, but there is also a risk of transmitting them from infected crops back to younger ones. Pest and disease identification, as well as, staff management are crucial at this time if disease levels are to be controlled within reasonable bounds. The general stages of mushroom cultivation can be seen in figure 2.

Once the crop becomes uneconomical it should be terminated. Compost is steam treated, at 60-70°C, for a minimum of twelve hours, before being removed from the cropping rooms in order to prevent the carry over of pests and pathogens to the next crop (Ingold and Hudson, 1993). The spent compost should then be removed within 24 hours of steaming.

Storage

Once harvested, mushrooms should be cooled quickly to 2°C. This cooler temperature reduces the rate of respiration and weight losses are kept to a minimum (Rinker, 1993). After harvest, mushrooms should be transported as soon as possible in

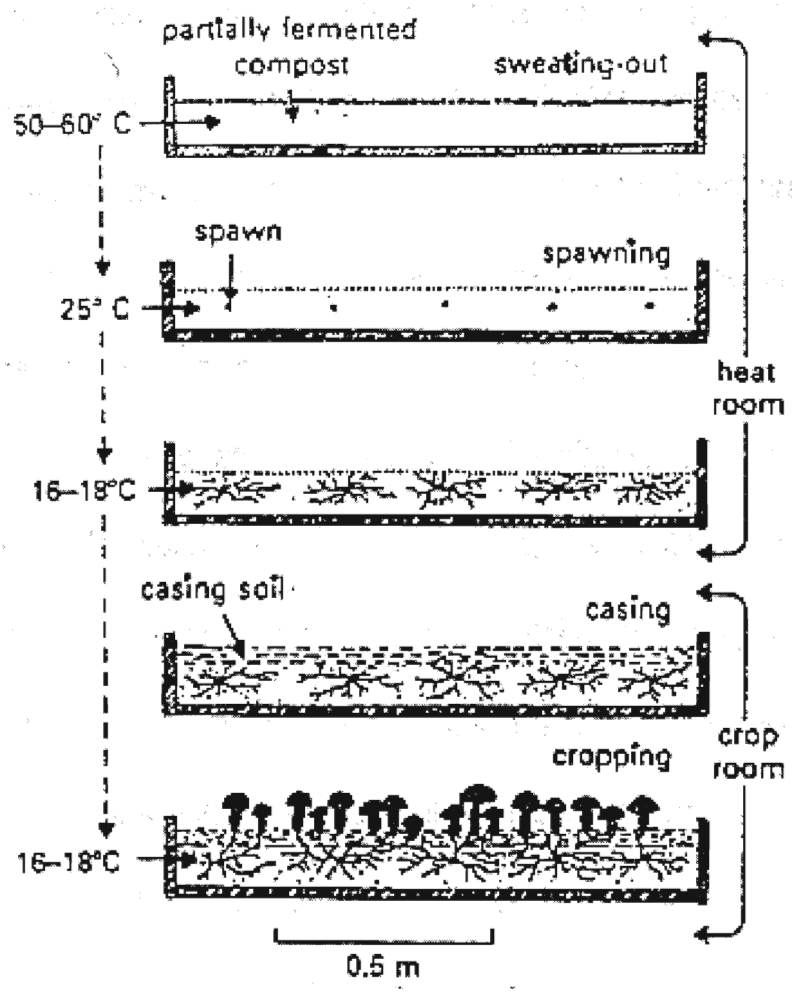

Figure 2. The indoor stages of mushroom cultivation.
The compost in this diagram is in wooden trays. However, whether compost is packed in bags, trays or in bins on shelves, the indoor stages of mushroom cultivation are the same (Ingold and Hudson, 1993).

climate controlled trucks to the market, where they should be displayed on refrigerated shelves. Fresh mushrooms will remain in good condition for approximately 5 days if held near 0°C, 2 days at 5°C and only 1 day at 9°C (Rinker, 1993).

Mushrooms should always be handled gently to prevent bruising which decreases postharvest quality. In addition, mushrooms should not be washed after harvesting since efficient drying is difficult and any remaining surface moisture will promote the development of bacterial organisms which will also lower postharvest quality (Rinker, 1993). Once mushrooms are harvested they should not be handled again. The postharvest quality of mushrooms is therefore a result of good horticultural practices and storage techniques.

Fungal Flora of Mushrooms

Fungi are associated with all stages of *A. bisporus* cultivation. During composting, thermophilic and mesophilic fungi play an important role, utilizing simple organic compounds to leave macromolecules which are suitable for *A. bisporus* growth. These fungi include *Acremonium* spp., *Aspergillus fumigatus, Cladosporium* spp., *Doratomyces microsporus, Mucor pusilus, Penicillium oxalicum, Humicola grisea, Torula thermophila* and *Myriococcum* spp. (Eicker, 1980; Fergus, 1964, 1978; Harvey, 1982; Schisler, 1982). A number of fungi can colonize the casing or compost after pasteurization. Fordyce (1968) determined the relative numbers of microorganisms in compost throughout the growing and cropping phases. He found a number of mesophilic species of the genera *Aspergillus, Epicoccum, Fusarium, Monilia, Mucor* and *Penicillium* in compost during the first two weeks after spawning. Other compost molds, such as *Botryotrichum*

piluliferum, Chaetomium olivaceum, Chromelosporium fulva, Coprinus sp., *Doratomyces microsporus, Oedocephalum* sp., *Mortierella* sp., *Paecilomyces* sp., *Papulaspora byssina* and *Scopulariopsis fimicola*, may pose problems to the mushroom crop as pathogens or competitors (Harvey *et al.,* 1982). Some compost molds, such as *Trichoderma viride, Trichoderma koningii* and *Oidiodendron sindenia* may be antagonistic towards mushroom mycelium (Harvey *et al.*, 1982; Beyer 1978).

Fungal pathogens of *A. bisporus*

Verticillium spp.

One of the most common and serious diseases of *A. bisporus* is dry bubble disease, which is caused by *Verticillium fungicola* var. *fungicola* (Preuss) Hassebr. (syn. *Verticillium malthousei* Ware) (Fletcher *et al.,* 1989). Symptoms caused by *V. fungicola* vary and depend on the developmental stage of the mushroom at the time of infection. When infection occurs early, the mushrooms develop into small undifferentiated masses of tissue which may be covered in a fine grayish-white mycelial growth (Fletcher *et al.,* 1989). Spotting symptoms occur when more fully differentiated mushrooms are affected. Frequently, blue-gray spots occur which are 1-2 cm in diameter over the cap surface. These spots often have a yellow or bluish-gray halo around them (Fletcher *et al.,* 1989). The spots caused by *V. fungicola* can, however, vary in colour from pale brown to blue-gray. These spots are generally less shiny than those caused by bacterial blotch (Rinker and Wuest, 1994). North (1987) showed that the inoculum level of *V. fungicola*

influenced the time of symptom expression. When 5μL of a 2.0 x 10^6 spores/mL suspension were inoculated onto growing mushrooms, disease symptoms were observed at the time of harvest. When 5μL of a 2.0 x 10^5 spores/mL suspension were inoculated onto growing mushrooms, disease symptom expression was delayed until after harvest. It was suggested, therefore, that *V. fungicola* might develop on harvested mushrooms during storage or transport. Wong and Preece (1987) recovered *V. fungicola* from symptomless mushrooms and suggested that mushrooms could be picked only to have disease symptoms develop during transport or during display at retail outlets. In addition to these studies, Gandy (1972) demonstrated that the primary cause of spotting, as a result of fungal disease, on postharvest mushrooms was *V. fungicola.*

A few other species of *Verticillium* have been associated with disease in mushroom crops. *V. fungicola* var. *aleophilum* affects mushroom pilei on which it produces dark brown spots (Fletcher *et al.,* 1989). *V. psalliotae* Treschow also produces spotting symptoms on mushroom pilei similar to those caused by *V. fungicola* var. *fungicola* (Subramanian, 1983). Unlike *V. fungicola* var. *fungicola,* neither of these fungi produces distorted mushrooms.

The strategy for control of *Verticillium* disease is basically good farm sanitation and hygiene (Rinker and Wuest, 1994). A number of fungicides are used, however, *Verticillium* is generally resistant to the benzimidazoles such as benomyl (Rinker, 1997). Reducing the crops to three breaks can significantly reduce the incidence of this disease on commercial mushroom farms.

Trichoderma spp.

Some species of *Trichoderma* have been shown to cause cap spotting of mushrooms. Cap spotting usually occurs when there is extensive colonization of the wood of trays, especially on the mushrooms which develop near the edges (Fletcher *et al.,* 1989). The spots are characterized as being pale brown in colour, without a clearly defined edge, usually less than 5mm in diameter and numerous (Fletcher *et al.,* 1989). The spots may also be reddish or purple brown in colour and are sometimes confused with *Verticillium* spot (Rinker and Wuest, 1994). It is not known whether all species can cause spotting, although *T. koningii* Oudem. and *T. pseudokoningii* have both been associated with severe outbreaks.

Trichoderma atroviride produces browning and pitting on mushroom pilei (Mithani, 1999). In this study, the time to symptom development was directly correlated with inoculum concentration.

Dano (2000) reported that *T. viride* Pers.: Fr., *T. koningii* and *T. harzianum* biotype Th1 Rifai produce browning and pitting on caps of *A. bisporus*. The symptoms produced and time of expression varied between the different species. Mushrooms infected with *T. viride* developed dark brown spots, whereas, *T. harzianum* biotype Th1 infected mushrooms developed larger, light brown spots. Mushrooms inoculated with *T. koningii* developed reddish spots. Symptoms were first observed on mushrooms inoculated with *T. harzianum* biotype Th1 three days after inoculation and next on *T. viride* (day four). The development of disease on mushrooms inoculated with *T. koningii* was slower and less severe than *T. harzianum* biotype Th1 and *T. viride.*

Control of *Trichoderma* spp. depends on thorough sanitation and hygiene, good insect and mite control as well as a properly prepared compost. *Trichoderma* spp. will flourish in compost when excess carbohydrates are available (Rinker, 1997). The use of fungicides, for example benomyl, may also help to decrease inoculum levels on the farm and therefore reduce disease incidence.

Penicillium spp.

Several studies have found a number of *Penicillium* spp. to be associated with mushroom sporophores after harvest (Fordyce, 1968; Seth and Shandylia, 1976; Davenport, 1990). These fungi were recovered from symptomatic and healthy mushroom tissue with similar frequencies. Seth and Shandylia (1976) determined *Penicillium* spp. to be the most frequently isolated fungi from postharvest mushrooms. Fordyce (1968) tested the effect of these fungi on postharvest mushroom quality but did not find them to be pathogenic. Davenport (1990) reported *Penicillium* spp. to be associated with brown cap areas on postharvest mushrooms. She tested the pathogenicity of a number of *Penicillium* spp. and found they could produce light brown to dark brown lesions on the mushroom pilei.

Dactylium dendroides

Hypomyces rosellus (= *Cladobotryum dendroides* = *Dactylium dendroides)* (Albertini and Schweinitz: Fries) Tulasne is the causal agent of cobweb disease. Cobweb disease

appears as circular patches of white mycelium on mushroom beds (Rinker and Wuest, 1994). The mycelium also cover the mushrooms with a course white growth. These affected mushrooms then turn brown and rot. In addition to cobweb this fungus may cause brown or pink-brown spots on the sporophores, with a poorly defined edge (Fletcher *et al.,* 1989).

Mortierella baineiri

Mushrooms infected by *Mortierella baineiri* Cost. develop a disease known as shaggy stipe. The most characteristic symptom of this disease is the peeling of the stalk of the affected mushrooms, producing a shaggy appearance (Fletcher *et al.,* 1989). These mushrooms may also develop brown blotches on their caps, which are often surrounded by a yellow ring.

Aphanocladium album

Brown spots on the cap have been associated with the fungus *Aphanocladium album* (Preuss) Gams (Burton *et al,* 1987; Rinker and Wuest, 1994). This pathogen affects the cap, producing light brown to dark brown, roughly circular spots, up to 10mm in diameter.

Hormiactis alba

There have also been a number of reports of the fungus *Hormiactis alba* Preuss causing spotting symptoms on mushroom caps (Fletcher *et al.,* 1989; Rinker and Wuest, 1994). Spotting symptoms caused by *Hormiactis alba* are characterized by irregular brown spots or blotches, which are approximately 10mm in diameter. These spots can occur anywhere on the cap surface. *Hormiactis alba,* although a less common fungal pathogen, has been reported to cause significant yield loss to commercial mushroom production (Rinker and Wuest, 1994).

Bacterial Flora of Mushrooms

Numerous bacterial species are also associated with commercial mushroom production. Beelman (1987) reported that bacterial populations on mushrooms are responsible for the majority of mushroom spoilage. Bacteria not only increase mushroom browning, but also change the appearance and texture of mushrooms. Bacteria may lyse mushroom cells, releasing the cell contents which mix with bacterial mucilage to produce a thin sticky film on the mushroom surface (Gandy, 1985).

A wide range of bacterial species have been isolated from harvested mushrooms. Fluorescent, non-fluorescent and mucoid pseudomonads, *Moraxella-Acinetobacter, Bacillus, Micrococcus, Staphylococcus* and *Serratia* were recovered from harvested mushrooms in a study by Doores *et al.,* (1987). Mushroom deterioration was not attributed to any one bacterial species, although fluorescent pseudomonads constituted

approximately 65% of the bacteria isolated. They also observed that the total bacterial populations on mushrooms increase during storage from 1.9 x 10^7 to 2.5 x 10^{10} colony forming units/g after 10 days storage at 13°C.

Bacterial pathogens of *Agaricus bisporus*

Ewingella americana

Ewingella americana Grimont was identified as a pathogen of *A. bisporus* (Inglis *et al.,* 1996). This bacterium causes a browning disorder of the stipe called internal stipe necrosis (Richardson, 1993; Inglis, 1995).

Janthinobacterium agaricidamnosum

A bacterium, *Janthinobacterium agaricidamnosum* Lincoln, was also recently discovered, which causes a soft rot disease of *A. bisporus* (Lincoln *et al.,* 1999). This disease has caused localized devastation of *A. bisporus* crops in England. Other symptoms, included pitting and sticky blotches on mushroom pilei. A disease showing similar symptoms has also been reported from cave farms in France. This bacterium represents a novel species within the genus *Janthinobacterium.*

Pseudomonads

Members of the genus *Pseudomonas* are most frequently the cause of disease in the cultivated mushroom, *A. bisporus.* *Pseudomonas tolaasii* Paine and *Pseudomonas gingeri* Preece and Wong are the cause of brown blotch (Paine, 1919) and ginger blotch (Wong *et al.,* 1982), respectively. *Pseudomonas agarici* Young produces drippy gill disease (Young, 1970) and an unidentified pseudomonad is associated with mummy disease (Schisler *et al.,* 1968). Rainey and Cole (1988) reported an unknown species of *Pseudomonas,* which causes an unusual disease of *A. bisporus.* Affected sporocarps were frequently malformed and displayed a characteristic constriction of the gill region of the cap. The stipes of these infected mushrooms had extensive tissue necrosis with profuse bacterial ooze. The causal organism was a weakly fluorescent, motile pseudomonad.

Pseudomonads have also been implicated in pit (Fletcher *et al.,* 1989). The cause of pit is, however, not yet established, although it is thought to be the result of a bacterial infection. The symptoms of pit include small, dark (often black) slimy pits on the caps of otherwise healthy mushrooms. These pits may be several millimeters in depth, are randomly distributed on the cap surface and range in numbers from one to more than ten per mushroom (Fletcher *et al.,* 1989). It is has been reported that the symptoms are initiated at a very early stage in the development of the mushrooms. Generally, pit does not appear until fairly late in the crop, most frequently on the third break. It is often associated with conditions of very high relative humidity and poor evaporation.

Pseudomonas tolaasii

Pseudomonas tolaasii is known as the cause of brown blotch disease of the cultivated mushroom, *A. bisporus.* This bacterium was first described by Tolaas (Tolaas, 1915). *Pseudomonas tolaasii* is commonly isolated from mushrooms and associated growing media and is considered a normal constituent of the microbiota (Fletcher *et al.,* 1989). Brown blotch occurs during mushroom production and generally begins as a surface discoloration on caps, which later develops into dark brown, sunken or pitted lesions. *Pseudomonas tolaasii* bears physiological properties similar to those of *P. fluorescens* biovar V but can be distinguished from it by its pathogenicity to mushrooms and by a 'white line' test (Wells *et al.,* 1996). In addition to blotch, *Pseudomonas tolaasii* can cause postharvest spotting (Wells *et al.,* 1996; Olivier *et al.,* 1978).

There are a number of ways to decrease the incidence of blotch. Environmental manipulations, such as control of relative humidity, temperature and ventilation, are essential for minimizing the incidence of blotch but do not guarantee success (Wong and Preece, 1982). Chlorinated compounds are commonly used, although higher concentrations often cause browning of mushroom caps (Royse and Wuest, 1980). Biological control has been investigated. Potential control agents have been isolated and shown to be active antagonists (Miller and Spear, 1995). Other antagonists, mixed with specific bacteriophages strongly aggressive towards *P. tolaasii* have shown a high degree of success against the development of blotch. However, at a later stage strains of *P. tolaasii* naturally resistant to the phage were isolated (Munsch and Olivier, 1995). Recently, Soler-Rivas *et al.* (1999a) reported that a cell-free crude extract of the White Line Inducing Principle (WLIP) could inhibit browning of mushrooms caused by *P.*

tolaasii. Furthermore, a suspension containing 1.6 mg/mL of pure WLIP was able to inhibit the symptoms of brown blotch induced by 7.6 X 10^6 cfu/mL of *P. tolaasii*. WLIP could therefore be a potential agent for preventing this disease in mushroom crops.

Tissue browning

The tissue browning which arises upon infection of mushrooms is due to the formation of melanin pigments. In *A. bisporus*, two monophenols are the main precursors of melanin: *L*-tyrosine and γ-glutaminyl-4-hydroxybenzene (GHB), both derived from the shikimate pathway (Sasaoka *et al.,* 1980). In the presence of tyrosinase, these monophenols are oxidized into their corresponding *o*-diphenols (3,4-dihydroxyphenylalanine (L-DOPA) and γ-glutaminyl-3,4-dihydroxyphenylalanine (GDHB), respectively). The *o*-diphenols are also substrates of the enzyme and oxidized into their corresponding *o*-quinones (dopaquinone and GBQ, respectively). The *o*-quinones are extremely unstable and rapidly polymerize to form DOPA-melanins and GHB-melanins. *Agaricus bisporus* melanins are heterogenous molecules with a wide range of colouration from yellow to black including red and purple (Jolivet *et al.,* 1998).

Several researchers propose tyrosinase has a role in the defense mechanism against fungal and bacterial pathogens, due to the properties of its products (Boekelheide *et al.,* 1980; Jolivet *et al.,* 1998). *o*-Quinones can link proteins and inactivate them or polymerize into melanins. Both *o*-quinones and melanins have a large bacteriostatic power. In fungi, melanins appear to be important in conferring resistance to microbial attack (Hegnauer *et al.,* 1985). They can also have an antibiotic effect against antagonistic organisms. The main role of fungal melanins is probably to provide

protection against hydrolytic enzymes, such as glucanases and chitinases, thus preventing cell wall lysis (Bell and Wheeler, 1986). In addition tyrosinases might also be involved in spore formation and stability (Jolivet *et al.,* 1998).

Materials and Methods

Introduction

In this study bacteria and fungi, which may be responsible for the disease, were isolated from the postharvest lesions. These bacteria and fungi were then used in a series of pathogenicity trials in an attempt to reproduce the postharvest spotting symptom. The pathogenicity of *Pseudomonas* spp was then looked at, specifically. These pseudomonads were further identified and grouped based on their electrophoretic protein profiles. Finally, the presence of plasmid DNA was then compared in these strains.

Isolations

Microorganisms were isolated from the unusual postharvest lesions on caps of the commercial mushroom, *A. bisporus*, obtained from the commercial mushroom farm. Isolations were performed from the discoloured lesions on the day the samples were collected, by peeling back the top layer of mushroom tissue and excising tissue blocks from the edges of the spots. The tissue blocks were then either placed directly onto agar plates or streaked onto agar plates. Several different media were utilized, during the isolations (appendix 1). Tissue blocks were also excised from non-spotted tissue and similarly placed onto agar plates or streaked onto agar plates. Single bacterial colonies were subcultured after 24 hours growth at 25°C onto agar plates containing the same medium. Fungi were subcultured after several days growth at 25°C onto the same medium. The fungi were identified to genus level using identification keys (Barnett,

1960; Barnett *et al.,* 1990; Malloch, 1981). Bacteria were differentiated based on their ability or inability to fluoresce on Pseudomonas Agar F. The frequency of occurrence of each of the microorganisms recovered was then determined by dividing the number of positive isolations for that organism by the total number of isolations performed.

Diseased mushrooms were obtained from the commercial mushroom farm. Sample 1 was collected in May, 1998 and contained 8 mushrooms which all displayed the characteristic postharvest spotting disease. Of these mushrooms a total of 36 lesions were sampled. Two blocks of tissue were excised from each of the discoloured lesions. One tissue block from each of the lesions was placed onto a Malt Agar plate and one tissue block was smeared onto a plate containing Pseudomonas Agar F. Ten tissue blocks were also excised from non-spotted tissue and similarly placed onto Malt Agar plates or smeared onto Pseudomonas Agar F and served as controls.

Sample 2 was collected in June, 1998. This sample also contained 8 mushrooms. Fifteen lesions were sampled from this group of mushrooms. Two blocks of tissue were excised from each of the lesions. One tissue block was then placed onto a Malt Agar plate and one tissue block was smeared onto Pseudomonas Agar F. Controls included ten tissue blocks from non-spotted tissue used to inoculate the Malt Agar and Pseudomonas Agar F plates.

Sample 3 was obtained in August, 1998. This sample contained 11 mushrooms of which 27 lesions were sampled. Again two tissue blocks from each lesion were excised and placed onto Malt Agar or smeared on Pseudomonas Agar F plates.

Sample 4 was also obtained in August, 1998. This sample contained 9 mushrooms from which 73 lesions were sampled. Again isolations were performed similar to the first

three samples by excising two tissue blocks from each lesion and then inoculating Malt Agar or Pseudomonas Agar F plates.

To ensure that the media used were not selecting against a specific pathogen, which may have been responsible for the postharvest spotting disease, several additional media were then used. These included; Pseudomonas Agar F, King's B medium, Malt Agar, Malt Extract Agar, Luria Bertani medium, Potato Dextrose Agar and mushroom agar (appendix 1). These media were used in all subsequent isolations.

Sample 5 was collected in November, 1998. This sample contained 12 mushrooms from which 37 spots were sampled. Small tissue blocks were excised from the edge of the lesions and placed onto one of the seven media. Ten tissue blocks were used to inoculate each of the different media. A total of 70 tissue blocks were used in these isolations. In addition 14 tissue blocks were excised from non-spotted tissue and two tissue sections were placed onto each of the seven media employed in this trial. These tissue blocks served as controls.

Sample 6 was collected in May, 1999. This sample contained 15 mushrooms. From these mushrooms 61 lesions were sampled, similar to sample five. Again ten tissue blocks were used to inoculate plates containing the seven different media.

Sample 7 was collected in July, 1999 and contained 15 mushrooms. A total of 64 lesions were sampled. Isolations were performed from the discoloured lesions similar to samples 5 and 6. Ten tissue blocks were also used to inoculate each of the seven different media.

Glycerol Stock Cultures

Bacteria were stored by adding 150μL sterile glycerol to 850μL of overnight liquid cultures. Cultures were then transferred to sterile eppendorf tubes and stored at -70°C (Sambrook et al., 1989).

Storage of Fungal Cultures

Fungi were maintained on Malt Agar plates, stored at room temperature and subcultured every 4-6 weeks.

Fungal identification

All fungi were identified to genus level using identification keys (Barnett, 1960; Barnett *et al.,* 1990; Malloch, 1981).

Bacterial identification tests

Identification tests were performed according to Bergey's Manual of Determinative Bacteriology. Tests included: colour of fluorescence on Pseudomonas Agar F; growth at 41°C; growth at 4°C; levan formation from sucrose; arginine dihydrolase; oxidase reaction; denitrification; gelatin hydrolysis; starch hydrolysis; utilization of glucose, lactose, maltose, trehalose, 2-keto-gluconate, m-inositol, L-valine; nitrate used as nitrogen source; lecithinase (egg yolk reaction); gram stain; catalase. Pathogenicity on

mushrooms, the white line reaction and mushroom rapid pitting tests were further used to identify bacterial cultures.

White line test

The pathogenic form of *Pseudomonas tolaasii* is easily identified *in vitro* by a specific interaction which occurs between *P. tolaasii* and *P. 'reactans'*, a saprophytic bacterium commonly living on mushroom caps. When these two species are grown side by side on King's B medium or Pseudomonas Agar F a dense white precipitate can be seen between them. This is known as the white line reaction (Wong and Preece, 1979). This reaction is the result of a specific interaction between a diffusible compound produced by *P. 'reactans'* called the White Line Inducing Principle (WLIP) and tolaasin, the toxin produced by *P. tolaasii* which is responsible for the damage caused by the pathogen in the mushrooms (Rainey *et al.,* 1992). The mechanism by which the white line forms is not well understood.

Bacteria were tested for the white line reaction by streaking isolates approximately 2 cm apart on Pseudomonas Agar F plates (Wong and Preece, 1979). Within 3 days of growth at 25°C a white precipitate formed between positive cultures. This reaction could be intensified by placing the cultures at 4°C. All isolates were tested against *Pseudomonas tolaasii* ATCC 33618 (type culture) and *Pseudomonas 'reactans'* ATCC 14340 (American Type Culture Collection, Rockville, MD). Each isolate was tested against each ATCC culture two times.

Mushroom Rapid Pitting Test

Freshly harvested mushrooms were inoculated with 24 hour bacterial cultures using a sterile inoculating loop to smear bacteria on cap surfaces or sterile wooden toothpicks for wound-puncturing bacteria directly into the cap tissues (Wong and Preece, 1979; Wells *et al.,* 1996). The area smeared was approximately 1 cm in diameter. Each isolate was tested on three mushrooms by the smear technique and three mushrooms by the puncture technique. Inoculated mushrooms were stored in lidded plastic dishes overnight at 25°C and then placed at 4°C. Observations were taken daily, up to seven days, for lesion development. Wounded but uninoculated controls were included for each test, as well as, positive controls inoculated with *Pseudomonas tolaasii* ATCC 33618. Wound punctures that developed water-soaked lesions with darkened margins were considered positive. Smear lesions that became slightly discoloured but not sunken were considered weak reactions. Darkened and sunken lesions were rated as severe.

Pathogenicity trials

Pathogenicity experiments were conducted at the Mushroom Research Facility (MRF), of the University of Guelph, at the Horticultural Research Institute of Ontario, Vineland Station, ON.

Mushroom Production

Phase I compost was obtained from a commercial mushroom farm. It was pasteurized and conditioned in bulk at MRF. Approximately 25 kg of Phase II compost was spawned with 225 g of spawn (Sylvan 130, an offwhite hybrid strain), placed in plastic trays, measuring 45 x 57 x 23 cm, pressed to a depth of 23 cm and covered with plastic. The compost temperature during spawn run was maintained at 25°C with the carbon dioxide and relative humidity of the air at 3000 ppm and 95%, respectively. After 14 days of vegetative growth, the compost surface of each tray was cased with a wet mixture of sphagnum peat moss, buffered with lime to a pH of 7.4-7.6. Casing was applied to a depth of approximately 5 cm. Ten days after casing, compost temperatures were lowered to 20°C and carbon dioxide levels lowered to 1000 ppm to initiate pins. All crop phases were computer controlled using a system developed by Fancom (Fancom, Panningen, the Netherlands) (figure 3).

Experimental design

Bacteria and fungi, which were isolated from the postharvest lesions, were selected at random. Fungi were cultured on Malt agar plates for approximately three weeks at 25°C. Spore suspensions were made by placing 5mL aliquots of sterile tap water containing Tween 20 onto each plate. The culture dishes were gently agitated for 15 minutes and

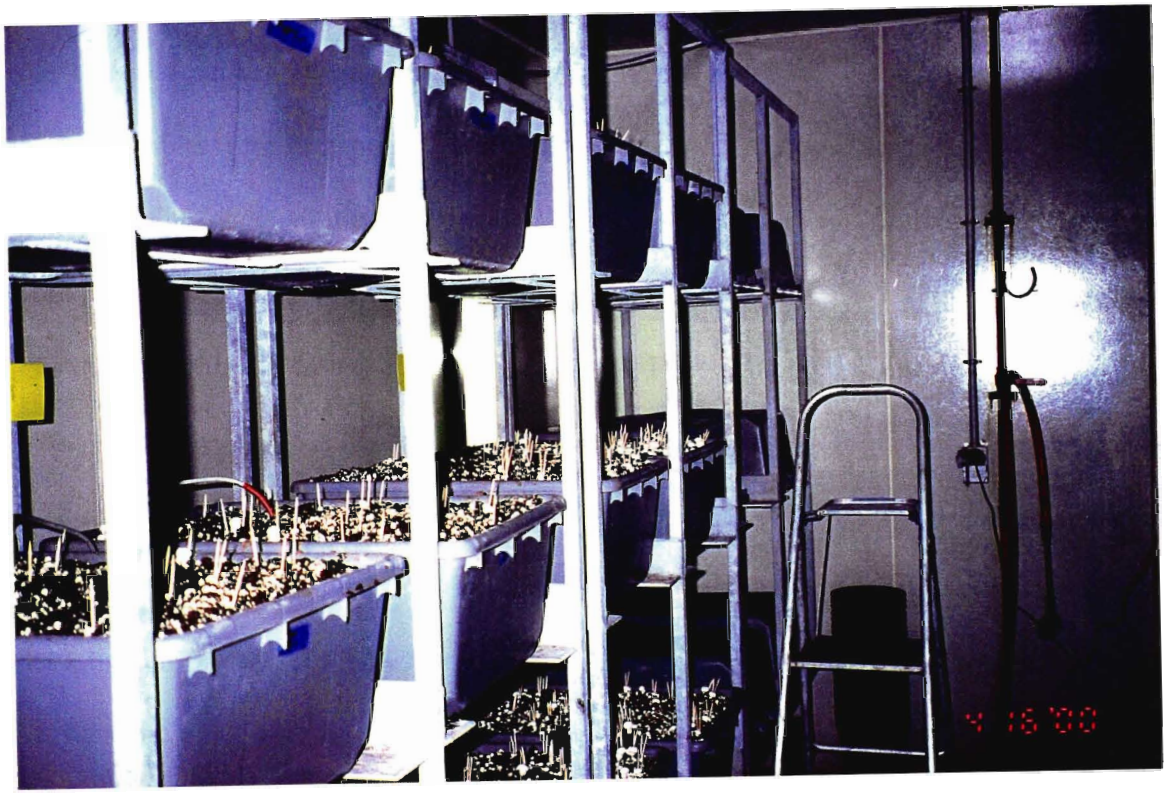

Figure 3. Growing room at the Mushroom Research Facility. The arrangement of trays used to grow mushroom crops for use in the pathogenicity trials is shown. One half of the growing room is represented in this figure.

then the liquid harvested into sterile tubes. A 100μL aliquot of the spore suspension was then diluted 10 fold and spore concentrations determined using a haemocytometer. Spore concentrations were verified by dilution plating. Spore concentrations were then adjusted to the proper concentration required for a given experiment.

Bacterial strains were cultured overnight at 25°C in liquid culture. Concentrations of the bacterial cultures were determined by taking the absorbance reading at 600nm. All concentrations were determined in cfu/mL. The concentrations were verified by dilution plating. The concentration of the bacterial culture was then adjusted to the specific concentration required for a specific experiment.

An unidentified yeast species was also tested for pathogenicity to mushrooms. The yeast was grown for two days in Malt Extract broth.

Each organism was evaluated for pathogenicity on three trays. Trays were located on three different carts and on three different levels of the growing room, in a randomized complete block design (RCBD), to account for environmental variation within the room. Mushrooms were inoculated three days prior to harvest, at the pin stage of development (except where noted) (figure 4). The experiments were repeated for each of the three breaks of the mushroom crop.

Harvested mushrooms were placed cap side up in 12-ounce styrofoam tills and stored at 8°C. Observations were taken daily for up to 7 days for lesion development on the mushroom caps.

For small-scale pathogenicity trials, small bags containing 5kg compost were used instead of trays to grow mushroom crops. The small-scale pathogenicity experiments

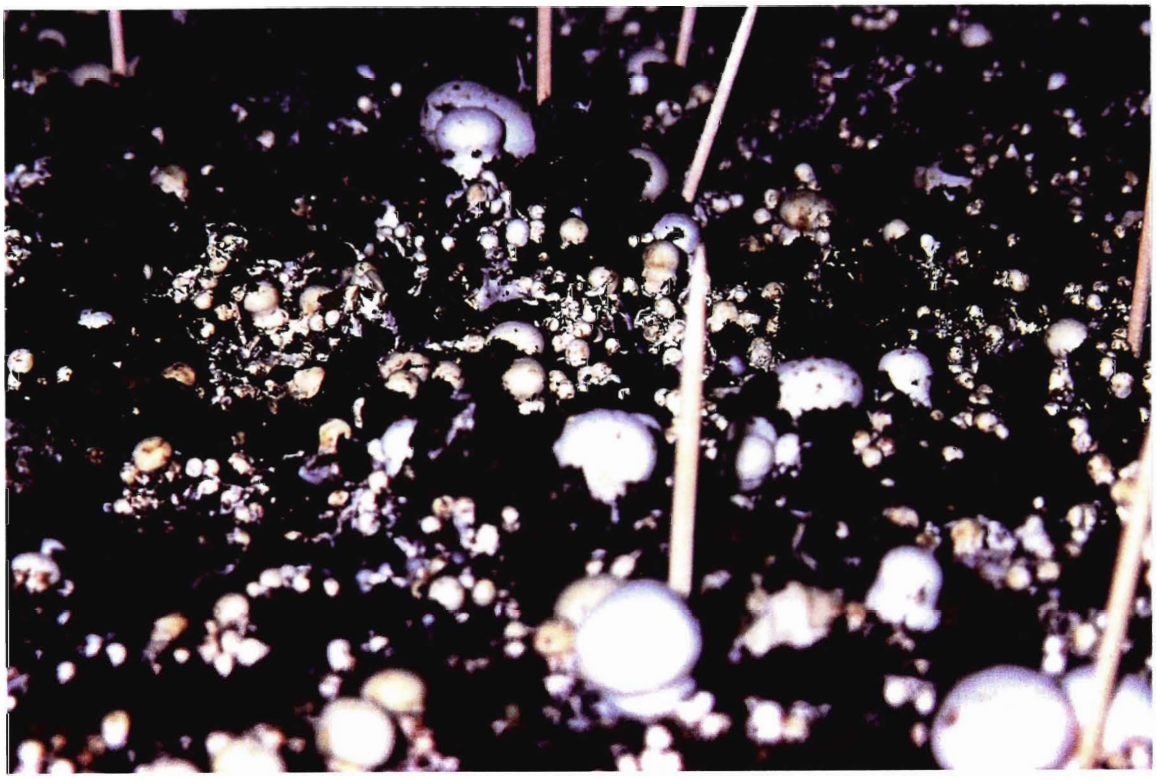

Figure 4. Mushrooms at the pin stage of development. Mushrooms were inoculated with randomly selected bacteria and fungi, three days prior to harvest, at the pin stage of development. Inoculated mushrooms were marked with wooden toothpicks.

were conducted similar to the large-scale experiments. Mushrooms were inoculated, harvested and stored similar to the large-scale experiments.

In the first large-scale pathogenicity trial a total of eleven organisms isolated from the postharvest lesions were evaluated for pathogenicity. Each isolate was tested on three trays. A total of 36 trays were used in this trial in the growing room. The remaining three trays were inoculated with one of three ATCC cultures (*P. 'reactans'* ATCC 51314, *P. tolaasii* ATCC 51309, both of which were isolated from a similar postharvest spotting disease in Pennsylavania (Wells *et al.,* 1996) and the *P. tolaasii* type culture ATCC 33618) for breaks 2 and 3.

Pathogenicity of *Pseudomonas* spp. isolated from the postharvest lesions

Two subsequent trials were conducted in order to further evaluate the pathogenicity of the pseudomonads isolated from the postharvest lesions. The first was a large-scale trial, which tested the pathogenicity of six *P. tolaasii* isolates on 18 trays at MRF. The second trial used 24 5kg bags in which to grow mushroom crops. Six different *P. tolaasii* isolates were tested for their pathogenicity in this trial.

Pathogenicity of fungi isolated from the postharvest lesions

In order to rule out the possibility that a fungus was responsible for the postharvest spotting disease, several fungal isolates were tested for their pathogenicity to mushrooms. A small-scale pathogenicity trial was conducted at MRF in which 10 5kg bags were used

to grow mushroom crops. Isolates representing five fungal genera were then chosen at random for use in this trial and used to inoculate mushroom pilei at concentrations of 10^4 and 10^6 spores. Concentrations were chosen based on results obtained by North (1987) and Davenport (1990). The yeast species was used to inoculate mushrooms at an unknown concentration. Fungi tested included; *Verticillium fungicola, Chromelosporium fulvum, Trichoderma* sp., *Penicillium* sp. and a yeast sp. The *Verticillium fungicola* used in this trial was not isolated from the postharvest lesions but from a mushroom with dry bubble disease collected from another commercial mushroom farm. This fungus was tested for pathogenicity to mushrooms since *Verticillium fungicola* has been associated with postharvest spotting of *A. bisporus* (North, 1987).

Pathogenicity of *P. tolaasii* at various inoculum levels

Once the postharvest spotting symptom of interest was found to be produced by *P. tolaasii* it was then necessary to try to determine at what inoculum level this occurred. A large-scale experiment was then conducted in which a *P. tolaasii* isolate (now designated strain 57) from the postharvest lesions was used to inoculate mushroom pilei at six different inoculum doses. Mushroom pilei were inoculated with either 10^1, 10^3, 10^5, 10^7, 10^9 or 10^{11} cfu of *P. tolaasii* (57). Fifty mushrooms were inoculated at each dose, for each of three breaks (except for 10^{11} cfu, which was used for break three mushrooms only). Similar to other experiments, the mushrooms were inoculated three days prior to harvest. Observations were taken daily for both pre- and postharvest symptom development.

Comparison of the pathogenicity of *P. tolaasii* strains

Since the spotting symptoms produced by the *P. tolaasii* strain (57) isolated from the postharvest lesions appeared to be unique, the pathogenicity of this strain was then compared to the *P. tolaasii* type culture (ATCC 33618) and *P. 'reactans'* (ATCC 14340). In addition, the pathogenicity of strain 57 was compared to *P. tolaasii* ATCC 51309 and *P. 'reactans'* ATCC 51314, since these pseudomonads were isolated from a similar postharvest spotting disease in Pennsylvania (Wells *et al.,* 1996). Mushrooms were inoculated three days prior to harvest. Ten mushrooms were inoculated for each of three doses (10^1, 10^3 and 10^5 cfu) for each of three breaks of the mushroom crop. One region of the mushroom pileus was inoculated with *P. tolaasii* strain 57 and another region inoculated with one of the four ATCC cultures. These mushrooms were then harvested into sterile 12-ounce styrofoam tills and stored at 8°C. Observations were taken of these mushrooms daily for up to seven days for lesion development. In a subsequent trial the pathogenicity of strain 57 was again compared to that of the type culture (ATCC 33618). In this trial each of the bacterial cultures was used at a dose of 10^5 cfu (since it was found in the earlier trial that ATCC 33618 will not produce any symptoms on mushroom pilei at a concentration lower than this) and each was used to inoculate one side of the same mushroom pileus. Mushrooms were harvested and stored similar to the previous trial. Daily observations for lesion development were performed for up to seven days after harvest.

Identification and grouping of bacterial isolates by analysis of their electrophoretic protein patterns

Once the *P. tolaasii* from the postharvest lesions was shown to differ in its pathogenicity to mushroom pilei from that of the *P. tolaasii* type culture (ATCC 33618), it was then necessary to determine how these strains differ at the molecular level. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins was utilized for the further identification and grouping of bacteria. Polyacrylamide gel electrophoresis of cellular proteins has been used for the classification and identification of bacteria to the sub-species level (Kersters and DeLey, 1975). Electrophoretic polypeptide patterns were therefore obtained for a number of *Pseudomonas* spp. isolated from the postharvest lesions as well as for the *Pseudomonas tolaasii* type culture (ATCC 33618). All electrophoretic separations were repeated four times. A total of eight gels containing cellular proteins of 3 strains of *P. tolaasii* shown to cause the postharvest spotting symptom and cellular proteins of 3 strains of *P. tolaasii* shown to produce typical blotch symptoms were analyzed. The cellular proteins of the *P. tolaasii* type culture were also run on each of these gels. A total of 12 isolates of each *P. tolaasii* strain were analyzed. In addition the electrophoretic polypeptide patterns were obtained for the two *Pseudomonas* cultures which were isolated from the postharvest spotting disease observed in Pennsylvania (*P. tolaasii* ATCC 51309 and *P. 'reactans'* ATCC 51314) (Wells *et al.,* 1996). Polypeptide patterns were then compared by computing correlation coefficients from scanning densitometric tracings (using the AIS Image

Analysis System) and then clustering the strains, by the unweighted average pair group method, using the Cluster Analysis Program, Winstat® Statistics for Excel.

Whole-cell protein isolation

Two mL overnight cultures of bacteria (grown in Luria Bertani medium) were used to inoculate 500mL of Luria Bertani medium. These cultures were then incubated for 48 hours at 25°C. Cells were harvested by centrifugation at 12 000xg and the resulting cell pellet washed twice with sodium phosphate buffered saline (appendix 2). Washed cells (50mg wet weight) were then suspended in 0.9mL of sample treatment buffer (appendix 2). Then 0.1mL 20% SDS (sodium dodecyl sulfate) was then added and the suspension mixed again. The cell suspension was then heated at 95°C for 10 minutes in a heating block. The samples were then centrifuged at 10 000xg for 10 minutes and the supernatant transferred to sterile microcentrifuge tubes. The tubes were stored at -20°C.

Determining Protein Concentration

Whole cell protein concentrations were determined using the Bio-Rad protein assay based on the Bradford assay (Bradford, 1976). Five µL of sample proteins were diluted with 795µL distilled H_2O and to this was added 200µL of Bio-Rad reagent dye. Two minutes after the dye was added 4mL of distilled H_2O was then added to the tubes to give a final volume of 5mL. The protein concentrations were then determined by comparing the absorbance readings (at 595 nm) to those of known concentrations of Bovine Serum Albumin (BSA) treated with reagent dye. A graph of absorbance vs. concentration was

constructed for the standards. The concentration of protein in the samples was then made by interpolating from the curve.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The SDS-PAGE procedure of Laemmli (1970) was employed to separate and analyze proteins. A 4% stacking gel (appendix 2) and 11% separation gel (appendix 2) were used with the Bio-Rad Protein 11 minigel apparatus.

Before each electrophoretic run 60mL of electrophoresis buffer (appendix 2) was diluted in 240mL dH_2O and used to fill the upper and lower buffer chambers of the electrophoretic tank.

Prior to electrophoresis, 2 X sample buffer (appendix 2) was added to the protein samples (equal volumes) and heated at 95°C for 4 minutes. Protein samples were then loaded onto the 4% stacking gel using a microsyringe. A constant voltage of 150 volts was then passed through the gel for a period of one hour. After this time the proteins have migrated through the gel at a distance proportional to their electrophoretic mobility.

Coomassie blue staining

To visualize protein bands on the SDS-Polyacrylamide gels they were then stained with Coomassie brilliant blue R-250. Gels were placed in a staining solution containing 0.25% Coomassie blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid. The gels were slowly rotated overnight. The gels were then destained in a solution containing 40% methanol and 10% acetic acid until the desired background was obtained.

Gels were stored in 10% acetic acid and photographed using Kodak T-Max 100 black and white film.

Plasmid DNA

Mamoun *et al.* (1997) reported a direct correlation between the presence of plasmid DNA and increased virulence of *P. tolaasii*. Based on their results it was hypothesized that plasmid DNA may be responsible for the difference in pathogenicity among *P. tolaasii* strains of this study. In this study plasmid DNA isolations were performed from both the *P. tolaasii* type culture (ATCC 33618) and a *P. tolaasii* strain from the postharvest lesions (57). The isolation of plasmid DNA from these strains was attempted four times.

Plasmid DNA mini-screen

The Birnboim and Doly method (1979) was used to isolate plasmid DNA from the bacterial cultures. Bacteria were grown in 2mL of Luria Bertani medium for 24 hours and harvested by centrifugation (8000xg for 5 minutes). The supernatant was removed and the cell pellet resuspended in 1mL of TE (appendix 2). To the cell suspension was then added 2mL of alkaline lysis solution (appendix 2) and the tubes were then inverted 20 times to obtain a uniform mixture. The tubes were then incubated in a water bath at 60°C for 30 minutes. Six mL of a 25:24:1 solution of phenol:chloroform:isoamyl alcohol was then added, the tubes vortexed briefly to achieve complete emulsification and then centrifuged at 8000xg for 12 minutes at 4°C. The upper aqueous phase was then

transferred to clean tubes and 1 volume of a 24:1 solution of chloroform:isoamyl alcohol was added. The tubes were again vortexed briefly and then centrifuged at 8000xg for 10 minutes. The upper aqueous phase was again transferred to clean tubes and to this was added 2.5 volumes of ice-cold isopropanol. The DNA was allowed to precipitate for 1 hour at -20°C. The tubes were then centrifuged at 12 000xg for 10 minutes, the supernatant removed and the DNA resuspended in 20μL of TE.

Restriction Endonuclease Digestion

Restriction enzymes were used at a stock concentration of 10 units/μL. Restriction enzymes used were: *Sma*I, *Xba*I, *Pst*I and *Bam*HI. The DNA sample and the enzyme were suspended in a total volume of 20μL with the buffer specified by the manufacturer for the enzyme. In addition, 0.25μL of a 10mg/mL solution of RNAse A was added to the reaction mixture. Samples were then heated at 37°C for a minimum of 2 hours to allow complete digestion of DNA.

Agarose Gel Electrophoresis

Plasmid DNA was resolved electrophoretically in a gel of 0.7% (w/v) agarose prepared in TAE buffer (appendix 2) (Sambrook *et al.,* 1989). Two μL of 6x loading buffer (appendix 2) was added to 10μL of plasmid DNA. The samples were then heated at 65°C for 2 minutes and then loaded onto the gel. Electrophoresis was carried out at 80

volts for 2 hours. The DNA was then stained for 20 minutes in ethidium bromide (1μg/mL).

Statistical Analyses

Chi-square tests and correlation analyses were performed using Minitab Statistical Software for Windows. Wald's Chi-square analysis was performed using SAS (Statistical Analysis System). Dendograms based upon the polypeptide data were constructed using the WinSTAT® Statistics for Excel Cluster Analysis Program.

Results

Isolations

The most frequently recovered microorganisms from the 313 lesions sampled were *Pseudomonas* spp (87%) (Table 1). Other bacteria were recovered from 30% of the postharvest lesions. The most frequently recovered fungal genus was *Penicillium* (16%). Other fungal genera recovered at low levels in the postharvest lesions included yeast (6%), *Trichoderma* spp. (3%), *Chromelosporium fulvum* (1%) and *Verticillium fungicola* (0.4%).

A Pearson product moment correlation analysis was then carried out on the isolation data to determine if a relationship existed between the postharvest lesions and the microorganisms isolated (appendix 3). From this analysis the correlation between the postharvest lesions and the microorganisms was determined (Table 2). The *Pseudomonas* spp. were found to be strongly correlated to the postharvest lesions ($r = 0.951$, $p = 0.001$). Yeast spp. were also strongly correlated with the postharvest symptom ($r= 0.894$, $p=0.004$).

Isolations were also performed from other symptomatic and non-symptomatic areas on the *Agaricus bisporus* pilei, which had the unusual postharvest spotting disease (appendix 4). A number of bacteria and fungi were recovered from the asymptomatic areas. A total of 34 isolations were made from these areas. Of the 34 isolations *Pseudomonas* spp. were most frequently recovered (38%). Bacteria (9%), *Trichoderma* sp. (3%) and *Chromelosporium fulvum* (3%) were also recovered from these areas at a low level.

Table 1. Mean percent recovery, per tissue block, of fungi and bacteria and total number of mushrooms and isolations therefrom, collected from the commercial mushroom farm in Ontario from May 1998 to July 1999.

Collection date	# of mushrooms	Total # of isolations spots		**% Recovery Per Isolation**						
				Pseudomonas spp.	Other bacteria	*Penicillium* spp.	Yeast spp.	*Trichoderma* spp.	*Chromelosporium fulvum*	*Verticillium fungicola*
May 1998	8	36	72	92	6	44	6	6	0	0
June 1998	8	15	30	93	33	0	0	0	7	0
August 1998	11	27	54	96	4	4	0	4	0	0
August 1998	9	73	146	100	29	15	33	0	0	0
November '98	12	37	70	89	16	11	0	11	3	0
May 1999	15	61	70	56	44	20	0	0	0	0
July 1999	15	64	70	86	77	17	0	0	0	3
	<u>78</u>	<u>313</u>	<u>512</u>	**87**	**30**	**16**	**6**	**3**	**1**	**0.4**

- <u>underlined</u> = total
- **bold** = mean
- note: percent recovery can be greater than 100% since more than one organism may be recovered from a tissue block.

Table 2. Correlation between the postharvest lesions and the bacteria and fungi recovered from them, as well as, the correlation between asymptomatic areas and the bacteria and fungi isolated therefrom.

Isolate	Postharvest lesions:		Asymptomatic areas:	
	*r	p	*r	p
Pseudomonas spp.	0.951	0.001	0.995	0.019
Other bacteria	0.363	0.226	0.866	0.101
Penicillium spp.	0.471	0.160	0.000	0.000
Yeast spp.	0.894	0.004	0.000	0.000
Trichoderma spp.	0.000	0.824	-0.500	0.105
Chromelosporium fulvum	0.195	0.317	1.000	0.000
Verticillium fungicola	0.000	0.934	0.000	0.000

- *r = Pearson product moment coefficient of correlation

The correlation between asymptomatic areas on the mushroom pilei and the organisms isolated from these areas was also determined (appendix 3, Table 2). None of the organisms was found to be strongly correlated with the asymptomatic areas.

White Line and Mushroom Rapid Pitting Tests

Strains of fluorescent pseudomonads isolated from the discoloured lesions on the postharvest mushrooms were assayed for pathogenicity using the Mushroom Rapid Pitting Test (Wong and Preece, 1979; Wells *et al.,* 1996). In addition, these strains were assayed for the white line reaction against *Pseudomonas tolaasii* ATCC 33618 (type culture) and *Pseudomonas 'reactans'* ATCC 14340 (Wong and Preece, 1979). The results of these assays are shown in Table 3.

Of the 395 isolates of fluorescent pseudomonads recovered from the postharvest lesions, 169 were white line positive against *Pseudomonas 'reactans'* ATCC 14340 and were designated *P. tolaasii.*

A simple correlation analysis was carried out in order to determine the relationship between pathogenicity to mushrooms and white line reactivity (appendix 5). There was a strong positive correlation (r=0.999) between isolates of pseudomonads which were mushroom rapid pitting test positive and those that were white line reactive when grown in close proximity to *Pseudomonas 'reactans'* ATCC 14340. In contrast there was a strong negative correlation (r=-0.825) between those isolates that were mushroom rapid pitting test positive and those isolates which were white line negative.

Table 3. Pathogenic properties of strains of fluorescent pseudomonads isolated from postharvest mushroom lesions

Sample	Total strains isolated	Mushroom rapid pitting test positive	White line positive with ATCC 14340	White line positive with ATCC 33618	White line negative
May'98	60	11	11	0	49
June'98	38	11	11	0	27
Aug.'98	50	8	8	0	42
Aug.'98	73	12	12	0	61
Nov.'98	57	38	38	0	19
May'99	58	41	43	0	15
July'99	59	46	46	0	13
	395	**167**	**169**	**0**	**226**

- bold = total

Pathogenicity trials

The postharvest spotting symptom of interest was reproduced by those bacteria which were identified as *Pseudomonas tolaasii* (Table 4 and appendices 6-a, 6-b and 6-c). No other bacteria or fungi tested in this trial produced the symptom. In addition, the ATCC cultures which were isolated from a similar postharvest spotting problem in Pennsylvania, (ATCC 51314 and ATCC 51309) (Wells *et al.,* 1996), did not produce the postharvest symptom of interest. The majority of the mushrooms remained asymptomatic after inoculation. Control inoculated and non-inoculated mushroom pilei also remained asymptomatic. Subsequent re-isolations from mushrooms, which developed the postharvest lesions of interest, revealed the presence of *Pseudomonas* spp.

To test the hypothesis that disease symptom and organism were independent a Chi-square test was performed. This test was performed for the three breaks of the mushroom crop (appendix7). The results were as follows; $\chi^2 = 20.005$, df = 5 and $p < 0.01$. Based on these results, the null hypothesis, that disease symptom and organism are independent, can be rejected. This test provides evidence of an association between *Pseudomonas tolaasii* and the postharvest spotting disease.

Pathogenicity of *Pseudomonas* spp. isolated from the postharvest lesions

Pseudomonas tolaasii isolates were shown to be able to produce the postharvest spotting symptoms on inoculated mushroom pilei (Tables 5 and 6 and figure 5). In the large-scale pathogenicity trial up to 12% of the inoculated mushrooms developed the symptom of interest on postharvest mushrooms. In the small-scale pathogenicity

Table 4. Results of the large-scale pathogenicity experiment for randomly selected bacteria and fungi isolated from the postharvest lesions for three breaks of the mushroom culture.

Isolate	1st break	2nd break	3rd break	Total spotted	# mushrooms inoculated	% spotted
Penicillium sp. (L27-10-1)	0	0	0	0	330	**0**
Trichoderma sp. (L27-2-1)	0	0	0	0	330	**0**
Yeast sp. (LII-7-1)	0	0	0	0	330	**0**
Pseudomonas putida (L12-1-1)	0	0	0	0	330	**0**
Pseudomonas sp. (L1598-5-1)	0	0	0	0	330	**0**
Pseudomonas tolaasii (LII-2-1)	0	30	0	30	330	**9**
P. tolaasii (L27-8-1)	0	26	0	26	330	**8**
P. tolaasii (L1597-1-1)	0	45	0	45	330	**14**
P. tolaasii (L27-3-1)	0	56	0	56	330	**17**
P. tolaasii (L12-8-1)	0	20	11	31	330	**9**
P. tolaasii (L27-12-1)	0	35	11	46	330	**14**

- note: data represents only those mushrooms which develop the postharvest spotting symptom of interest
- mushrooms inoculated three days prior to harvest and stored at an average temperature of 8°C

Table 5. Results of a large-scale experiment testing the pathogenicity of *Pseudomonas tolaasii* cultures isolated from the postharvest lesions on *Agaricus bisporus* pilei collected from the commercial mushroom farm.

P. tolaasii Isolate	Break	# mushrooms inoculated	Harvest symptoms	Postharvest symptoms	Total diseased	% postharvest	% diseased
*-4-2	1	150	0	0	0	0	0
	2	150	0	18	18	12	12
	3	150	3	16	19	11	13
*-5-2	1	150	0	0	0	0	0
	2	150	0	12	12	8	8
	3	150	2	12	14	8	9
*-6-1	1	150	1	0	1	0	0.7
	2	150	0	10	10	7	7
	3	150	1	12	13	8	9
*-1-3	1	150	0	0	0	0	0
	2	150	0	11	11	7	7
	3	150	5	17	22	11	15
*-2-2	1	150	0	0	0	0	0
	2	150	2	10	12	7	8
	3	150	2	18	20	12	13
*-3-1	1	150	0	0	0	0	0
	2	150	0	11	11	7	7
	3	150	1	12	13	8	9
		2700	17	159	176		

- mushrooms inoculated three days prior to harvest
- mushrooms stored at an average temperature of 8°C
- underlined = total

Table 6. Results of a small-scale experiment testing the pathogenicity of *Pseudomonas tolaasii* cultures isolated from the postharvest lesions on *Agaricus bisporus* pilei from mushrooms collected from the commercial mushroom farm.

P. tolaasii isolate	Break	# mushrooms inoculated	Harvest symptoms	Postharvest Symptoms
L1597-1-1	1	40	3	1
	2	40	5	0
	3	40	3	2
L27-3-1	1	40	2	2
	2	40	2	1
	3	40	2	3
L27-12-1	1	40	1	4
	2	40	2	3
	3	40	1	2
L27-3-2	1	40	0	5
	2	40	1	3
	3	40	0	2
LI-1-3	1	40	3	0
	2	40	3	0
	3	40	0	0
LII-31-4	1	40	0	0
	2	40	1	0
	3	40	0	0
Control	1	48	1	0
	2	48	2	1
	3	48	1	0
		*720	*29	*28

- underlined = total; * = total does not include control inoculated mushrooms
- control = Luria Bertani medium
- mushrooms inoculated three days prior to harvest
- mushrooms stored at an average temperature of 8°C

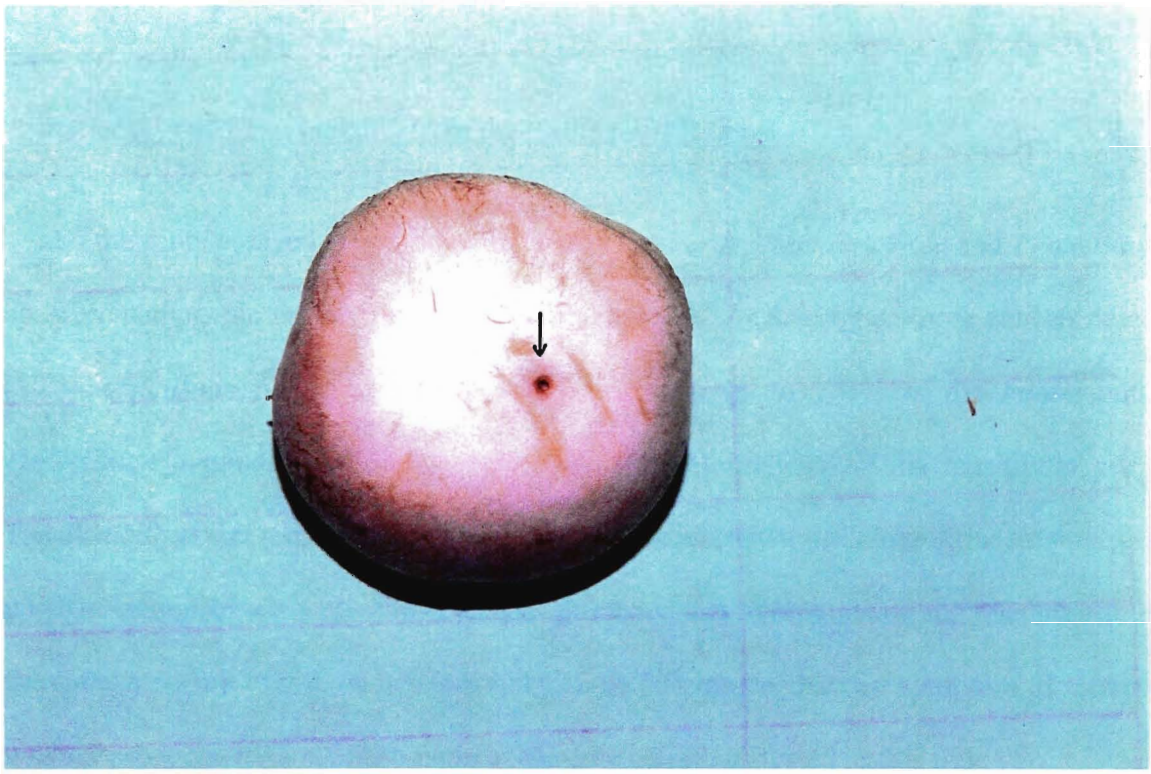

Figure 5. Postharvest spotting on mushroom cap inoculated with *P. tolaasii* (strain 57). Arrow indicates area of inoculation.

experiment, four of the *P. tolaasii* isolates produced the postharvest symptom of interest. However, two of the *P. tolaasii* cultures produced golden brown discolourations on preharvest mushrooms at a very low level. They did not produce any symptoms on postharvest mushrooms.

Pathogenicity of fungi isolated from the postharvest lesions

Of the fungi assayed for pathogenicity only the *Verticillium fungicola* and *Penicillium* sp. were pathogenic on the mushroom pilei (Table 7). At the higher spore concentration (10^6) *Verticillium fungicola* produced symptoms on up to 100% of the mushrooms. *Verticillium fungicola* produced golden brown discolourations on the mushroom pilei. The *Penicillium* sp. was less pathogenic and produced brown discolourations on pre- and postharvest mushrooms. No other fungus produced any symptoms on the mushrooms at the concentrations tested. It is important to note that the postharvest symptom of interest was not produced by any of the fungi at the concentrations tested in this trial.

Pathogenicity of *P. tolaasii* at various inoculum concentrations

The postharvest spotting symptom of interest was reproduced at concentrations of 10^1, 10^3 and 10^5 cfu (Table 8, appendix 8). At concentrations above 10^7 symptoms appeared on preharvest mushrooms and resembled the typical brown blotch lesions associated with this pathogen. At the lower concentrations time to symptom development varied with inoculum concentration (appendix 8). At 10^1 cfu symptoms appeared six or seven days after harvest. When mushrooms were inoculated with 10^3 cfu the symptoms appeared

Table 7. Pathogenicity of several fungal genera isolated from the postharvest lesions on *Agaricus bisporus* pilei.

Isolate	[spore]/ 100µl	Break	# mushrooms inoculated	Harvest symptoms	Postharvest symptoms	Total
Verticillium fungicola	10^4	1	10	2	1	3
		2	10	0	1	1
		3	10	1	5	6
	10^6	1	10	6	1	7
		2	10	5	4	9
		3	10	1	9	10
Penicillium sp.	10^4	1	10	2	0	2
		2	10	1	0	1
		3	10	3	0	3
	10^6	1	10	1	1	2
		2	10	4	0	4
		3	10	3	0	3

- note: pathogenicity of *Chromelosporium fulvum,* yeast and a *Trichoderma* sp. were also tested but are not shown in the table since they failed to produce any symptoms on mushroom pilei.
- mushrooms inoculated 3 days prior to harvest
- mushrooms stored at an average temperature of 8°C

Table 8. Results of the pathogenicity trial for *Pseudomonas tolaasii* (57), showing the numbers of mushrooms which developed blotch or postharvest spotting after inoculation, at various concentrations, onto mushroom pilei.

Concentration (cfus)	# mushrooms inoculated/ break	Total # Mushrooms Inoculated	Break 1 Symptoms	Break 2 Symptoms	Break 3 Symptoms	Mean # of Symptoms
10^{11}	50	50	NA	NA	50	**50**
10^{9}	50	150	50	50	50	**50**
10^{7}	50	150	50	50	50	**50**
10^{5}	50	150	12*	29*	13*	**18**
10^{3}	50	150	19*	43*	37*	**33**
10^{1}	50	150	1*	5*	2*	**3**
Control	50	150	0	0	0	**0**

- db = dark brown; * = postharvest spotting
- **bold** = mean
- control = Luria Bertani media
- mushrooms inoculated three days prior to harvesting and stored at an average temperature of 8°C
- NA = not applicable

approximately four days after harvest, similar to what was observed at the commercial mushroom farm. At 10^5 cfu the symptoms developed within approximately one day of harvest. Control mushrooms remained asymptomatic.

The results of the Wald's Chi-square analysis can be seen in appendix 9. The effect of dose was found to be positive (0.7021 +/- 0.0480). The significance of dose is seen by Wald's Chi-square = 214.1782 (with 1 df) and $p < 0.0001$. The data therefore suggests an effect of dose.

Comparison of the pathogenicity of *Pseudomonas tolaasii* strains

The pathogenicity of the *P. tolaasii* (strain 57) from the postharvest lesions was then compared to the *P. tolaasii* strains obtained from the American Type Culture Collection. Mushroom pilei were co-inoculated with strain 57 and one of the ATCC cultures at several doses. Only strain 57 produced the postharvest spotting symptom of interest (Table 9).

In a subsequent trial the pathogenicity of strain 57 was compared to the *P. tolaasii* type culture (ATCC 33618). Both bacteria were used at a concentration of 10^5 cfu (since it was found in the earlier trial that the type culture does not produce symptoms when used at inoculum levels below 10^5 cfu). Again only the *P. tolaasii* isolated from the postharvest lesions produced the postharvest spotting symptom of interest (Table 10). Symptoms produced on the *A. bisporus* pilei differed between strains (figure 6). At this concentration 18% of the mushrooms developed the postharvest spotting symptom of interest in the area of the mushroom pilei inoculated with strain 57. Only 2% of the

Table 9. Side by side comparison of the pathogenicity of *Pseudomonas* spp. co-inoculated onto the same mushroom pilei.

Culture	Concentration (cfus)	# mushrooms inoculated	**Postharvest** (57)	**Spotting:** (ATCC)
57 + 51314	10^1	30	2*	0
	10^3	30	5*	0
	10^5	30	14*	0
57 + 51309	10^1	30	1*	0
	10^3	30	7*	0
	10^5	30	18*	7 (lb)
57 + 14340	10^1	30	0	0
	10^3	30	9*	0
	10^5	30	7*	0
57 + 33618	10^1	30	0	0
	10^3	30	20*	0
	10^5	30	8*	6(gb)
		<u>360</u>	<u>91</u> (25)	<u>13</u> (4)

- lb = light brown; gb = golden brown; * = postharvest symptom
- mushrooms inoculated 3 days prior to harvesting
- mushrooms stored at an average temperature of 8°C
- <u>underlined</u> = total; ( ) = percent total which develop postharvest spotting

Table 10. Side by side comparison of the pathogenicity of *Pseudomonas tolaasii,* isolated from postharvest lesions on *Agaricus bisporus* pilei, and the *Pseudomonas tolaasii* type culture (ATCC 33618) inoculated onto the same mushroom pilei at a concentration of 10^5 cfu.

Tray	# mushrooms inoculated	# with postharvest spotting (57)	# with postharvest spotting (33618)
1	50	7*	2 (golden brown)
2	50	5*	0
3	50	16*	1 (golden brown)
4	50	12*	0
5	50	3*	0
6	50	11*	2 (light brown); 1 (golden brown)
	300	54*	6
	50	**9** (18)	**1**(2)

- mushrooms inoculated 3 days prior to harvest
- mushrooms stored at an average temperature of 8°C
- underlined = total
- **bold** = mean; brackets = percent of total mushrooms which were spotted
- * = postharvest spotting symptom

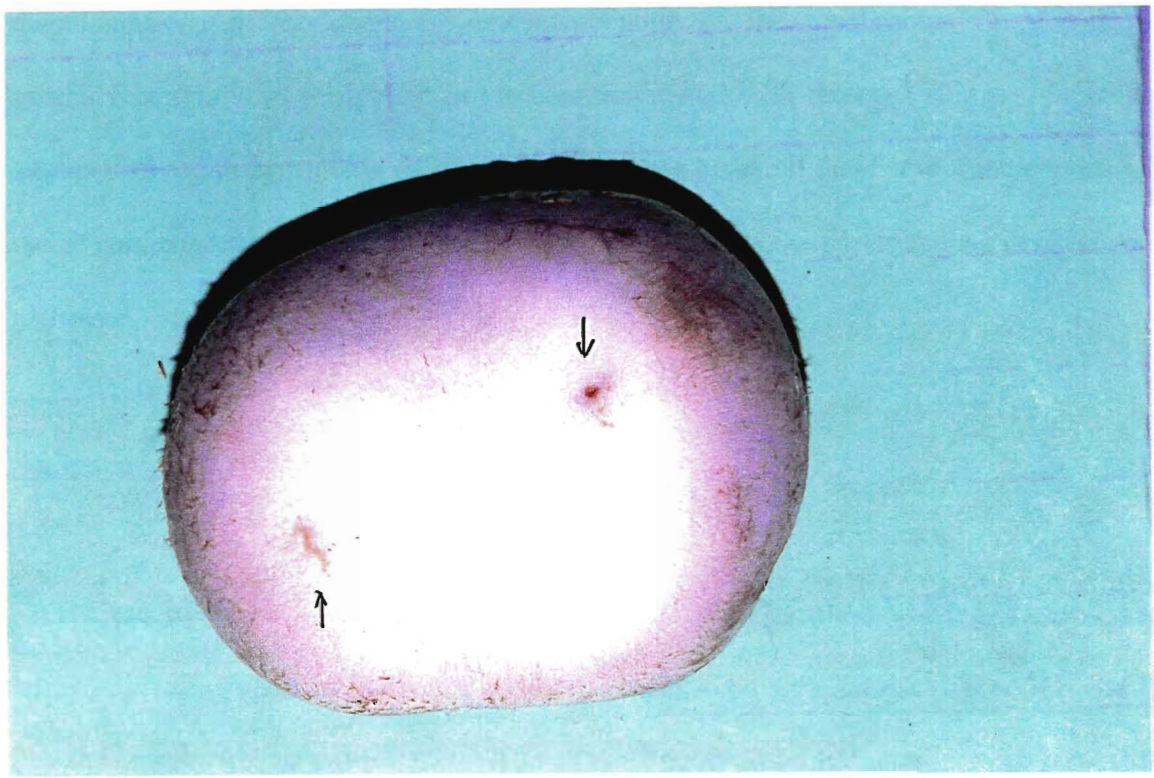

Figure 6. Co-inoculation of mushroom pilei with *P. tolaasii* strains. The mushroom pileus above developed the postharvest spotting symptom in the area inoculated with a *P. tolaasii* strain isolated from the postharvest lesions (strain 57) (right side of pileus, indicated by arrow). In contrast there is a light brown discolouration in the area of the mushroom pileus inoculated with the *P. tolaasii* type culture (ATCC 33618) (left side of pileus, indicated by the arrow).

mushrooms developed postharvest symptoms in the area inoculated with the type culture (ATCC 33618). These mushrooms developed golden brown or light brown discolourations in those areas. However, none of the mushrooms developed the postharvest symptom of interest in the area inoculated with the type culture. Since the strains differed in symptoms produced, on the same host and under the same conditions, the *P. tolaasii*, exemplified by strain 57, from the postharvest lesions was designated a pathovar.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The electrophoretic polypeptide patterns of the pseudomonads were compared. In terms of banding patterns the ATCC cultures appear to be very similar (figure 7). The *P. tolaasii* isolates from the postharvest lesions also appear to be very similar with respect to their gel electrophoretic protein patterns (figure 7). Numerous differences between the *P. tolaasii* isolates from the postharvest lesions and the ATCC isolates were observed. Protein patterns were compared further by computing correlation coefficients from scanning densitometric tracings (using the AIS Image Analysis System) and then clustering the strains, by the unweighted average pair group method, using the cluster analysis program (Winstat® Statistics for Excel). The results of this analysis can be seen in figure 8 and appendix 10.

The *P. tolaasii* strains isolated from the postharvest lesions were then compared. Cellular proteins were isolated from *P. tolaasii* strains which were shown to produce the postharvest lesions of interest on inoculated mushrooms, as well as, *P. tolaasii* isolates

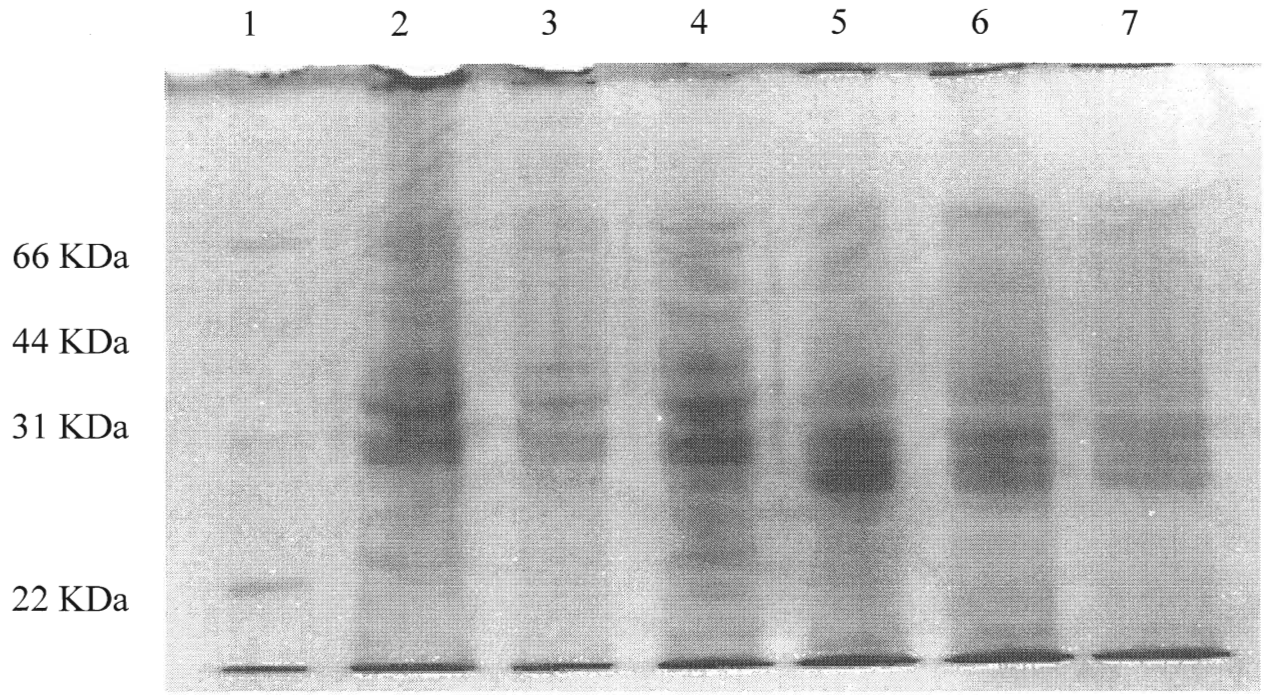

Figure 7. SDS-PAGE analysis of cellular proteins of representative members of *P. tolaasii* strains. Lane 1 contains a low molecular weight marker (Bio-Rad). Lane 2 contains the cellular proteins of *P. 'reactans'* ATCC 51314; lane3- *P. tolaasii* ATCC 51309; lane 4- *P. tolaasii* ATCC 33618; lanes 5, 6 and 7 contain the cellular proteins of three *P. tolaasii* strains isolated from the postharvest lesions.

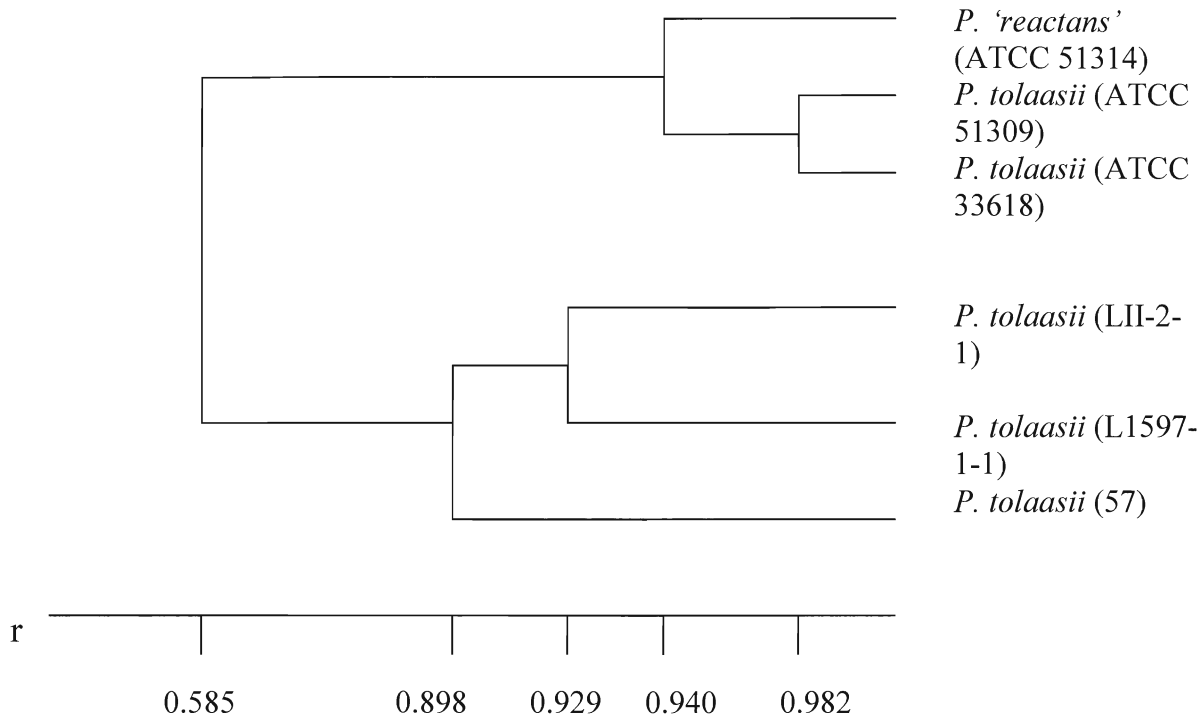

Figure 8. Dendogram based on electrophoretic protein patterns of fluorescent pseudomonads isolated from the postharvest lesions and three *Pseudomonas* spp obtained from the American Type Culture Collection.

which produced the typical brown blotch symptoms. These proteins were again analyzed via SDS-PAGE. The proteins from the type culture were also run on these gels. The results can be seen in figure 9. Protein patterns were again compared by scanning densitometric analysis. Strains were again clustered based on their electrophoretic protein patterns. The *P. tolaasii* isolates which cause the postharvest spotting symptoms on mushroom pilei were very similar in their electrophoretic protein patterns. The *P. tolaasii* isolates from the postharvest lesions which were shown to produce the typical blotch like symptoms on inoculated mushroom pilei were also found to be very similar in their electrophoretic protein patterns. A number of differences between these strains were observed. The results of these analyses can be seen in figure 10 and appendix 11.

Plasmid DNA Isolation

Since plasmid DNA has been implicated in the pathogenicity of *Pseudomonas tolaasii* (Mamoun *et al.,* 1997), an attempt was made to isolate plasmid DNA from the *Pseudomonas tolaasii* strain isolated from the postharvest lesions, as well as, from the type culture (*Pseudomonas tolaasii* ATCC 33618). Plasmid DNA isolations were carried out and then restriction endonuclease digestions performed using the restriction enzymes; *Sma*I, *Xba*I, *Pst*I and *Bam*HI. The DNA was then run on a 0.7% agarose gel (figure 11). As can be seen on the gel, neither strain 57 or the type culture contains plasmid DNA.

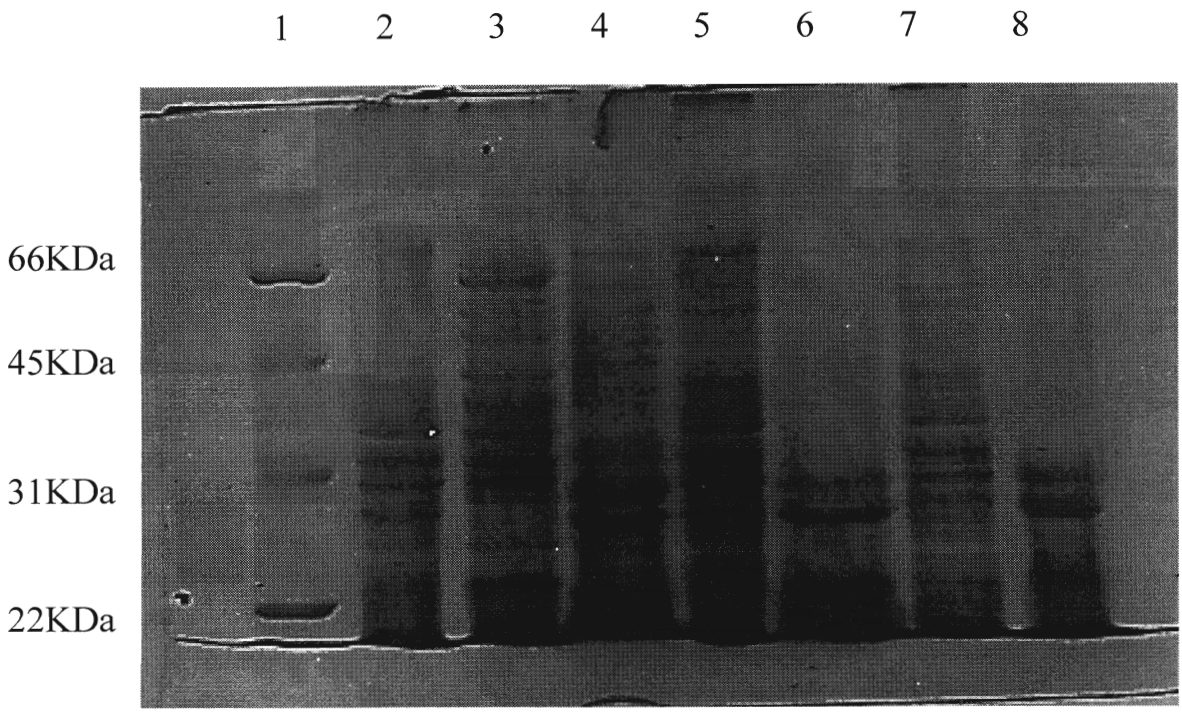

Figure 9. SDS-PAGE analysis of cellular proteins from *P. tolaasii* strains isolated from the postharvest lesions. Lane 1 contains a low molecular weight marker (Bio-Rad). Lanes 2, 5 and 7 contain the cellular proteins of *P. tolaasii* strains shown to produce the postharvest spotting symptom. Lane 3 contains the cellular proteins of the *P. tolaasii* type culture (ATCC 33618). Lanes 4, 6 and 8 contain the cellular proteins from *P. tolaasii* strains isolated from the postharvest lesions but which produce typical brown blotch symptoms when inoculated onto mushroom pilei.

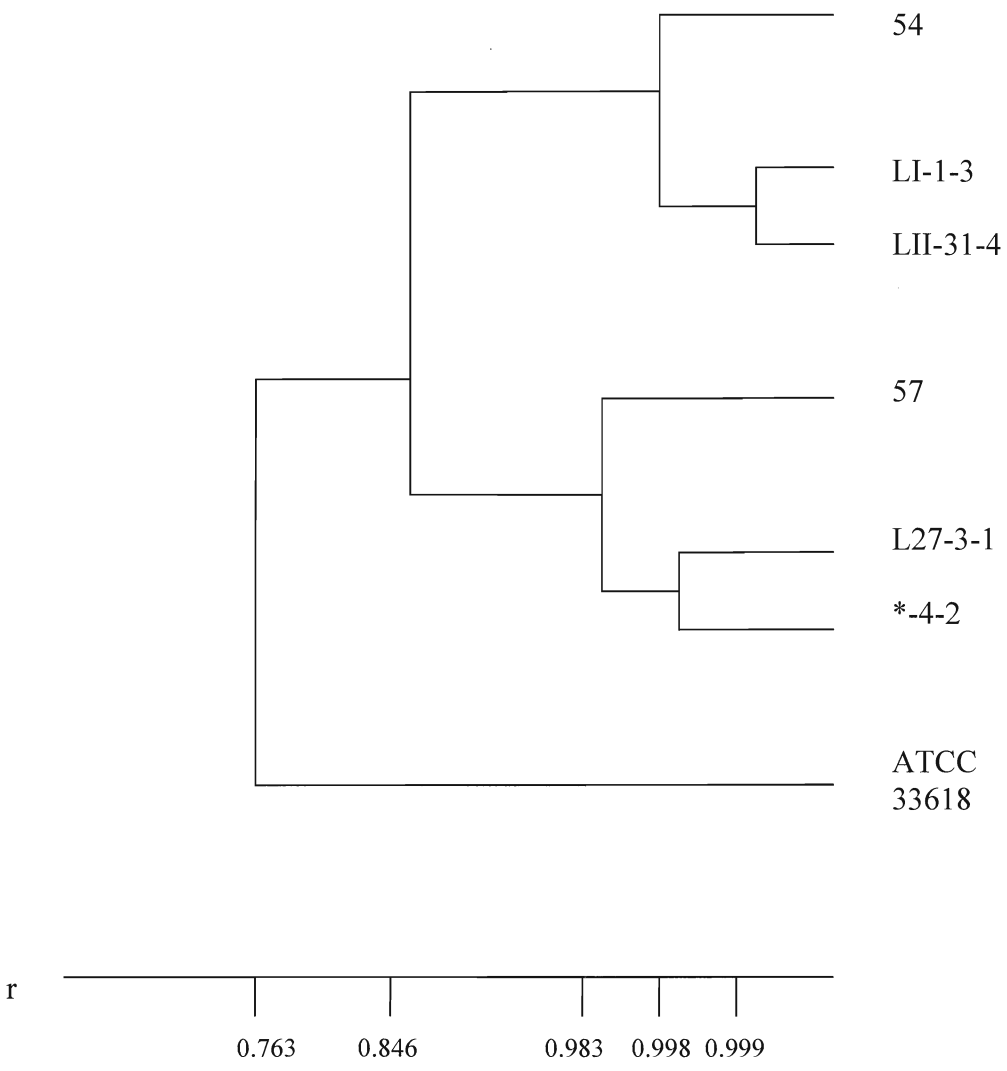

Figure 10. Dendogram derived from cellular protein profiles of seven pathogenic strains of *Pseudomonas tolaasii*. ATCC 33618 is the type culture. Strains 54, LI-1-3 and LII-31-4 were isolated from the postharvest lesions, but produced the typical brown blotch lesions when inoculated onto mushroom pile. Strains 57, L27-3-1 and *-4-2 are the pathogens responsible for the postharvest spotting disease.

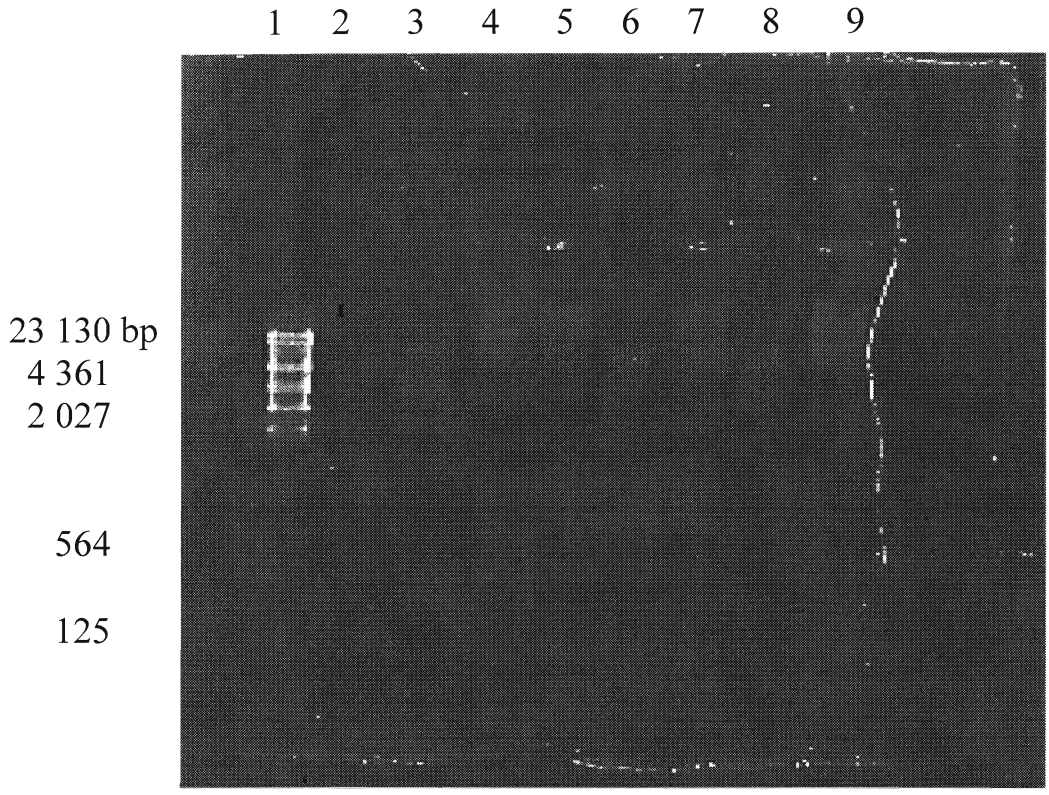

Figure 11. Restriction endonuclease digested DNA isolated from *P. tolaasii* strain ATCC 33618 and a *P. tolaasii* strain from the postharvest lesions (57). Lane 1 contains the DNA molecular weight marker II (Roche Biochemicals). Lane 2 contains DNA isolated from 57 digested with *Sma*I; Lane 3 contains DNA isolated from ATCC 33618 digested with *Sma*I. Lane 4 – 57 digested with *Xba*I; Lane 5 – 33618 digested with *Xba*I; Lane 6 – 57 digested with *Bam*HI; Lane 7 – 33618 digested with *Bam*HI; Lane 8 – 57 digested with *Pst*I; Lane 9 – 33618 digested with *Pst*I. None of the lanes contains plasmid DNA

Discussion

The postharvest spotting disease of *Agaricus bisporus,* observed at the commercial mushroom farm, was found to be caused by a novel pathovar of *Pseudomonas tolaasii*. Other bacteria and fungi recovered from these lesions were non-pathogenic or, if pathogenic, did not produce the postharvest spotting symptom of interest. Electrophoretic protein patterns of these *P. tolaasii* strains further demonstrate the uniqueness of this pathovar. Presence of plasmid DNA was not detected and is therefore not responsible for the difference in pathogenicity between the strains.

A number of fungi were recovered from postharvest mushrooms in this study, similar to what was observed in previous studies (Fordyce, 1968; Seth and Shandylia, 1976; Davenport, 1990). However, only a limited number of fungi were commonly recovered, in contrast to these previous studies. This may reflect improved sanitation, disease control practices and environmental control measures utilized at mushroom farms today. It is important to note, however, the use of excised tissue for fungal recovery, employed in this study, favoured fungi growing beneath the surface of the pileus. Fungi on the surface, whether saprophytic or pathogenic were not recovered.

Members of the genus *Penicillium* were the most frequently isolated fungi in this study. Various species of *Penicillium* are associated with mushroom culture and generally, they do not cause problems (Fletcher *et al.,* 1989). Its presence on postharvest mushrooms has been noted previously (Fordyce, 1968; Seth and Shandylia, 1976; Davenport, 1990). The effects of *Penicillium* spp. on mushrooms are not well understood. It is assumed that populations of *Penicillium* spp can increase in mushroom

crops grown where sanitation and air filtration is inadequate (Davenport, 1990). The type of compost and pesticide usage during cropping may also effect levels of *Penicillium* in a mushroom crop. Supplementation of compost and casing may increase *Penicillium* growth in the mushroom substrate by providing easily assimilated nutrients. The increased populations in the casing and compost would then increase the chance of *Penicillium* spores landing and perhaps growing on developing sporophores. Spores may also impact mushroom crops during packing and transportation since *Penicillium* spores are ubiquitous.

Penicillium spp are able to reside on and in mushroom tissue. Reisolation of this fungus was possible from inoculated areas after seven days storage. The *Penicillium* spp appear to be associated with brown areas, which suggests they can induce browning either as pathogens or saprophytes. A *Penicillium* sp was shown in this study to produce brown discolourations on pre- and postharvest mushrooms when inoculated onto pilei at 10^4 and 10^6 spores. These results confirm a study by Davenport (1990) which reported that *Penicillium* spp can produce brown spots on mushroom sporophores. However, *Penicillium* spp may not always induce symptoms, since it has also been recovered from asymptomatic areas on mushroom pilei (Davenport, 1990). The occurrence of *Penicillium* spp beneath the sporophore surface suggests active ingress into pilei tissue.

The common occurrence of *Penicillium* spp on *A. bisporus* sporophores demonstrates that these fungi can live successfully on the mushroom surface. It seems that *Penicillium* spp are facultative, being saprophytic with the potential to be pathogenic, albeit an opportunistic pathogen, on mushroom pilei.

The recovery of yeast was interesting, since little is known about their occurrence or effect on *A. bisporus.* Yeasts have been associated with the spoilage of many different food products (Pitt and Hocking, 1985). A *Candida* sp. has been previously isolated by Fordyce (1968). Davenport (1990) recovered a number of cellular and mycelial yeasts from postharvest mushrooms. These yeasts were isolated from brown areas on sporophores. However, their effects on mushroom quality are still unknown. The number of yeasts recovered from symptomatic areas suggests that they may pose spoilage problems on mushrooms and have pathogenic tendencies. However, their pathogenicity was not demonstrated in this study. It is likely that the yeasts are secondary invaders of the spotted tissue. Yeasts utilize simple carbohydrates for their nutrition (Moore-Landecker, 1996). It is possible that the spotted tissue, which has been digested by enzymes produced by the primary pathogen, allows them access to easily assimilated nutrients. More research is needed to determine the exact nature of the interaction between yeast and *A. bisporus.*

The incidence of *Trichoderma* spp on mushroom pilei appears to have decreased since earlier surveys (Fordyce, 1968; Seth and Shandylia, 1976; Davenport, 1990). This may be due to a change to the use of peatmoss at mushroom farms since 1968 when topsoil was the casing of choice (Wuest *et al.,* 1987). The introduction of benzimidazole fungicides may also have helped to reduce *Trichoderma* mushroom infections (Wuest *et al.,* 1987). Davenport (1990) showed a number of *Trichoderma* spp were pathogenic on sporophores, inducing brown to chocolate brown lesions. This has been reported by others (Seth and Shandylia, 1976; Harvey *et al.,* 1982). Dano (2000) demonstrated that three species of *Trichoderma* (*T. viride, T. koningii* and *T. harzianum* biotype Th1) were

pathogenic on mushrooms. Furthermore, at low spore concentrations ($<10^3$ spores) the symptoms would develop after harvest. Therefore, symptoms produced by *Trichoderma* spp may develop at the retail outlets causing reduced consumer appeal and return of the mushrooms. The occurrence of *Trichoderma* spp in this study was low and their pathogenicity was not demonstrated. However, these fungi still pose a threat to mushroom quality as shown by other researchers.

The *Chromelosporium fulvum* recovered from the postharvest lesions is commonly referred to as a peat mold (Eicker, 1989). This fungus causes cinnamon brown mold in mushroom crops. This mold rarely poses a threat to mushroom culture. This fungus was likely harboured in the peat moss and contacted mushrooms via water splashing. It was recovered from the postharvest lesions at a very low level and does not appear to be a threat to mushroom quality.

The presence of *Verticillium fungicola* on the sporophores was expected since others have reported that *V. fungicola* may proliferate on mushroom caps during storage (North, 1987; Davenport, 1990). *Verticillium fungicola* on postharvest mushrooms appears to originate in growing rooms. Its spores are not found in the normal air spora away from mushroom farms (Davenport, 1990). Infection of *A. bisporus* by this pathogen results in brown lesions, shattered stipes and undeveloped sporocarps (dry bubble disease). The degree of disease and the particular symptoms observed are dependent on the inoculum level and the point of development of the mushroom crop at the time of infection (North and Wuest, 1993). Similar to results obtained in previous studies (North, 1987; Davenport, 1990) the pathogenicity of *V. fungicola* was demonstrated in this study. Mushroom pilei developed golden brown discolourations on pre- and postharvest

mushrooms when inoculated with 10^4 and 10^6 spores. Even though the *V. fungicola* was recovered at a low level in this study, it nevertheless has a huge impact on mushroom quality.

It appears that a variety of fungi can reside on or in mushroom pilei. Many of these fungi are present at a low level and probably do not pose a significant threat to mushroom postharvest quality. Other fungi, such as, *Penicillium* spp were more frequently encountered and may contribute to the spoilage of harvested *A. bisporus*. Research to ascertain the effect of these fungi on mushroom caps is required for the development of effective measures to reduce postharvest spoilage.

Bacteria were commonly recovered from the postharvest lesions. This was not unexpected since a range of bacterial species have been isolated from harvested mushrooms (Doores *et al.,* 1987; Solomon, 1989; Davenport, 1990). In a storage trial lasting 10 days, Doores *et al.* (1987) recovered fluorescent, non-fluorescent and mucoid pseudomonads, *Moraxella-Acinetobacter, Bacillus, Micrococcus, Staphylococcus* and *Serratia*. The mushroom deterioration observed by Doores *et al.* (1987) was not attributed to any one bacterial species, although fluorescent pseudomonads constituted 65% of the bacteria isolated. In this study *Pseudomonas* spp were the most frequently isolated genera. Other bacteria were frequently isolated. However, these bacteria were not identified since their pathogenicity to mushrooms was not demonstrated. The relationship of these bacteria with *A. bisporus* sporophores should be looked at further to determine what effect they have on mushroom quality.

The postharvest spotting symptoms observed on mushrooms from the commercial mushroom farm, was caused by a novel pathovar of *P. tolaasii*. This pathovar was

identified and its relationship to known taxonomic groups, including *P. tolaasii* and *P. 'reactans'*, determined based on its physiological and biochemical properties, pathogenicity to mushrooms and composition of its cellular proteins. These data confirm earlier results, among them Olivier *et al.* (1978) and Wells *et al.* (1996) that *P. tolaasii* can cause postharvest symptoms on mushrooms.

Strains of fluorescent pseudomonads isolated from the discoloured lesions on the postharvest mushrooms were first tested for pathogenicity using the Mushroom Rapid Pitting Test (Wong and Preece, 1979; Wells *et al.,* 1996). In addition, these strains were tested for the White Line Reaction against *P. tolaasii* ATCC 33618 (type culture) and *P. 'reactans'* ATCC 14340 (Wong and Preece, 1979).

In the samples collected from May 1998 to August 1998, the pathogenicity of the pseudomonads was low, as was the number of pseudomonads which were white line positive against *P. 'reactans'* ATCC 14340. In contrast, there were a higher number of fluorescent pseudomonads which were pathogenic to mushrooms and white line positive against *P. 'reactans'* ATCC 14340 in the samples collected from November 1998 to July 1999. It is the tendency of *P. tolaasii* to undergo phenotypic variation, including loss of pathogenicity, upon subculturing which may account for the difference in pathogenicity of pseudomonads in the samples (Cutrie *et al.,* 1984).

Pseudomonas tolaasii can be found in two forms when grown on Pseudomonas Agar F or King's B media. The smooth form has non-fluorescent, opaque, semi-mucoid colonies and is pathogenic due to the production of tolaasin, whereas the rough form is fluorescent, translucent, non-mucoid and non-pathogenic (Cutrie *et al.,* 1984). The pathogenic form can switch spontaneously to a non-pathogenic form and stop production

of tolaasin. A functional copy of the *phe*N gene is required to maintain the pathogenic phenotype. Loss of function of this gene results in the switch to the phenotypic variant form. The phenotypic variant is unable to synthesize tolaasin.

Pseudomonads in the earlier samples were subcultured several times before being bioassayed for pathogenicity to mushrooms. In contrast, samples collected from November 1998 to July 1999 were subcultured only once and displayed a higher level of pathogenicity to mushrooms and white line positive reactions. By placing the pseudomonads into storage culture you can prevent their undergoing phenotypic variation to a non-pathogenic form.

The pre-harvest bacterial populations and factors that influence their size play an important role in the postharvest quality of mushrooms. In this study the postharvest spotting symptom of interest was reproduced on *Agaricus bisporus* pilei, when *Pseudomonas tolaasii* was used at concentrations of 10^1, 10^3 and 10^5 cfu. This markedly differed from the symptoms produced by the type culture, *Pseudomonas tolaasii* ATCC 33618. The type culture produced only very mild golden or light brown lesions on only a few of the mushroom pilei at a concentration of 10^5 cfu. Below this concentration all mushrooms remained asymptomatic. Based on these differences in pathogenicity between these strains, the *P. tolasii* isolated from the postharvest lesions appears to be a novel pathovar.

The minimal number of bacterial cells able to produce brown lesions after inoculation onto mushroom caps is difficult to estimate because of the instability of the bacterium. Doores *et al.* (1987) showed that aseptically harvested mushrooms contain approximately 10^7 cfu/gram of bacteria. Different concentrations have been reported as threshold

values, the proposed values ranging from $6.0x10^7$ to $1.0x10^8$ cells per cap (Preece and Wong, 1982), $2.7x10^6$ - 10^8 cfu/ml (Nair and Bradley, 1980), or from $7.7x10^4$-10^8 cells/cm^2 (Rainey *et al.,* 1992). This threshold value was found to be independent of size or age of the mushroom primordium or cap. The disease may be visible in crops at a very early stage or symptomless, although the pathogen is present. Mushrooms may then appear healthy at harvest only to develop symptoms during postharvest storage. This can even occur when they are stored at low temperatures, because *Pseudomonas tolaasii* possesses the ability of growing at low temperatures.

The *P. tolaasii* strains were further grouped based on their electrophoretic protein patterns. Comparisons of electrophoretic protein patterns can be a fast, easy and powerful tool for classification and identification of bacteria. Protein profiles can be used to identify bacteria to the sub-species level (Kersters and DeLey, 1975) Cellular protein profiles of strains of *P. tolaasii* isolated from the postharvest lesions showed these bacteria to be most similar to each other (89.8% proximity). The protein profiles of the ATCC cultures show these bacteria to be highly similar (94% proximity). The distance between the two clusters was found to be 58.5%.

The cellular protein profiles of the *P. tolaasii* strains responsible for the postharvest spotting disease was further compared to *P. tolaasii* strains which produce the typical brown blotch symptoms. Again the *P. tolaasii* strains responsible for the postharvest spotting disease clustered together (98.3% proximity). The *P. tolaasii* strains which produce blotch symptoms also clustered together (99.8% proximity). The distance between these clusters was found to be 84.6%. The strains of *P. tolaasii* isolated from the postharvest lesions bear closer resemblance to each other than to the ATCC cultures.

This difference in cellular protein profiles demonstrates differential gene expression in these bacteria. The difference in pathogenicity among these bacteria could be due to differences in amount of extracellular enzymes or toxins produced by these strains. These results further demonstrate the uniqueness of the pathovar.

The presence of plasmid DNA has been implicated in the pathogenicity of several *Pseudomonas* spp. Nigishi *et al.* (1993) demonstrated that plasmid pJTPS1 reduces or eliminates pathogenicity of *P. solanacearum*. In contrast Mamoun *et al.* (1997) reported that the presence of plasmid DNA is responsible for the high virulence of *P. tolaasii*. They investigated 15 wild-type *P. tolaasii* strains and found they all contained plasmid DNA. Strains which had lost the plasmid spontaneously showed a decrease in virulence which was not related to the shift from pathogenic (smooth) to non-pathogenic (rough) form described by others (Olivier *et al.,* 1978; Cutri *et al.,* 1984). Whereas, the chromosome-directed shift from the smooth to rough form induces a loss of pathogenicity, the loss of plasmid DNA reduces but does not suppress pathogenicity. The difference in pathogenicity was due to differences in toxin production and efficiency (Mamoun *et al.,* 1997). The wild-type strain produces larger amounts of more efficient toxin than strains which have lost the plasmid. This plasmid therefore mediates toxin production and efficiency but is not the only factor controlling these traits.

Based on the previous studies, plasmid DNA was postulated as the factor responsible for the virulence variation in the *P. tolaasii* strains of interest to this study. Although plasmid DNA was found to be responsible for the difference in pathogenicity in *Pseudomonas* spp previously (Nigishi *et al.,* 1993; Mamoun *et al.,* 1997) no plasmid DNA was found in either the *P. tolaasii* type culture (ATCC 33618) or *P. tolaasii* (57),

the pathogen responsible for the postharvest spotting disease. The difference in pathogenicity between these strains is therefore not due to the presence of plasmid DNA.

There are several factors which may explain the difference in pathogenicity observed between the *P. tolaasii* strains. It is possible that there are differences in the amount or type of tolaasin produced by these strains. Tolaasin has been shown to induce both browning, as well as, to activate mushroom tyrosinase (Soler-Rivas *et al.,* 1999b). It is produced only by the pathogenic form of *P. tolaasii.* When applied directly on mushrooms, it can reproduce the symptoms of the disease. It therefore appears to be a compound highly involved in the damage. There are a number of reasons which could account for differences in tolaasin production, such as, differences in growth kinetics between the strains, difference in the integrity of the *phe*N locus or presence of plasmid DNA. Presence of plasmid DNA , as mentioned previously, was not demonstrated in this study. It is also possible that the type of tolaasin is different. Tolaasin is synthesized by a non-ribosomal mechanism which requires a multi-enzyme complex and perhaps post-translational modification (Soler-Rivas *et al.,* 1999b). When this multi-enzyme complex adds amino acid residues to the structure, it does so with low specificity, which results in a variable amino acid composition. It is this variable amino acid composition which could effect the function of this toxin and therefore its effects on mushroom pilei.

Tolaasin is not the only compound responsible for bacterial blotch symptoms. Park *et al.* (1994) isolated a new compound from a *P. tolaasii* strain, inducing the symptoms of the disease on *A. bisporus* crops. After purification, this compound was characterized as an aminobenzene containing an amylamine group. More recently, Shirata (1996) described volatile compounds produced by the bacterium that are also part of the

infection process. These compounds, called tovsins, were different from tolaasin and able to induce browning and rotting on *Pleurotus ostreatus* (oyster mushroom) on both cultivating and packed fruit bodies.

Pseudomonas tolaasii also produces proteinases and lipases which are possibly involved in the infection process. *P. tolaasii* was found to produce an extra-cellular, monomeric, metallo-proteinase (Fairbairn and Law, 1986). The effect of this proteinase in mushroom infections is still unknown but it may help facilitate the damage caused in the mushroom (Baral *et al.,* 1995). Lipases are shown to facilitate bacterial infections by disrupting host membranes. *P. tolaasii* produces an extra-cellular heat-stabile, monomeric metallo-lipase (Baral and Fox, 1997). The role of this lipase during infection of mushrooms also requires further study.

Similar to tolaasin, differences in amounts of these toxins could account for the differences in pathogenicity observed between the *P. tolaasii* strains. Based on the results of the SDS-PAGE analyses, which showed differential gene expression in these strains, this seems possible.

Conclusions

The postharvest spotting disease of *Agaricus bisporus* was caused by a novel pathovar of *Pseudomonas tolaasii*. This pathovar differed from a *P. tolaasii* type culture in the symptoms produced on *A. bisporus* pilei. In addition, the pathovar differed from the type culture in its electrophoretic polypeptide patterns. The presence of plasmid DNA was not demonstrated in this study. The difference in pathogenicity between these strains is therefore not due to plasmid DNA.

Although the organism responsible for the postharvest spotting disease was identified, the control of this postharvest problem was not analyzed. This objective is therefore delegated to future research.

Future work should also focus on the types and amounts of toxins produced, analysis of *phe*N locus integrity and growth kinetics. These studies should help to elucidate how these strains differ from each other.

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Appendix 1

GROWTH MEDIA

Pseudomonas Agar F (Difco Laboratories, Detroit, MI.): 38g of Pseudomonas Agar F was suspended in 1L dH_2O containing 10g glycerol and then sterilized at 124°C for 30 minutes. Formula per liter: 10g tryptone; 10 proteose peptone; 1.5g dipotassium phosphate; 1.5g magnesium sulphate; 15g agar.

Malt Agar (Difco Laboratories, Detroit, MI.): 45g of Malt Agar was suspended in 1 liter dH_2O and sterilized at 124°C for 30 minutes. Formula per liter: 30g malt extract; 15g agar.

Malt Extract (Difco Laboratories, Detroit, MI.): 17.6g Malt Extract was dissolved in 1 liter dH_2O and sterilized at 124°C for 30 minutes. Formula per liter: 12.75g maltose; 2.75g Bacto Dextrin; 2.35g Bacto Glycerol; 0.78g Bacto Peptone.

Malt Extract Agar (Difco Laboratories, Detroit, MI.): 33.6g Malt Extract Agar was dissolved in 1 liter dH_2O. The media was sterilized at 124°C for 30 minutes. Formula per liter: maltose 12.75g; Bacto Dextrin 2.75g; Bacto Glycerol 2.35g; Bacto Peptone 0.78g; Bacto Agar 15g.

Luria Burtani broth: 10g tryptone; 5g yeast extract; 10g NaCl were suspended in 1 liter dH_2O and sterilized at 124°C for 30 minutes. For plates 15g agar was added before sterilization.

King's medium B (KB) (King *et al.*, 1954): 20.0g Proteose peptone; 10.0g glycerol; 1.5g K_2HPO_4; 1.5g $MgSO_4$-$7H_2O$; 15.0g agar were suspended in 1 liter dH_2O. The pH was adjusted to 7.2 and the medium sterilized by autoclaving at 124°C for 15 minutes.

Potato Dextrose Agar (Difco Laboratories, Detroit, MI.): 43g Potato Dextrose Agar was suspended in 1 liter dH_2O and sterilized at 124°C for 30 minutes.

Mushroom agar: Mushroom agar was prepared by homogenizing 500g of freshly harvested mushrooms in 500ml of dH_2O. Agar was then added to a concentration of 1.5% and the volume made up to 1 liter with dH_2O. The medium was then sterilized at 124°C for 15 minutes.

Appendix 2

LABORATORY REAGENTS

Cellular protein isolation reagents:

Sodium phosphate buffered saline: 0.01 M sodium phosphate buffer containing 0.8% NaCl (pH 7.3)

Sample treatment buffer: 0.062 M Tris-HCl buffer containing 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol; pH 6.8

SDS-PAGE reagents:

Separation gel: 3.7mL dH_2O; 2.5mL 1.5M Tris-HCl, pH 8.8; 3.7mL Acrylamide/Bis-acrylamide (30% stock solution) (Degas for 15 minutes at room temperature); 50µL 20% SDS; 50µL 10% ammonium persulfate; 5µL N,N,N',N'-tetramethylenediamine (TEMED). The separation gel was overlaid with n-butanol until polymerization was completed, then rinsed with distilled water before casting the stacking gel.

Stacking gel: 6.2mL dH_2O; 2.5mL 0.5M Tris-HCl, pH 6.8; 1.3mL Acrylamide/Bis-acrylamide (30% stock solution) (Degas for 15 minutes at room temperature); 50µL 20% SDS; 50µL 10% ammonium persulfate; 10µL TEMED.

Electrophoresis buffer: 60g Tris; 288g glycine; 20g SDS were dissolved in 4 liters dH_2O.

2 X sample buffer: 125mM Tris-HCl, pH 6.8; 20% glycerol (v/v); 4% SDS (w/v); 10% 2-mercaptoethanol; 0.01% bromophenol blue

Plasmid DNA isolation reagents:

TE: 10 mM Tris; 1 mM EDTA (pH 8.0)

Alkaline lysis solution: 3 g Sodium lauryl sulfate; 5 mL of 3 M NaOH in 100 mL of 0.05 M Tris (pH 12.0)

TAE buffer: 40 mM Tris; 20 mM acetic acid; 1 mM EDTA (pH 8.0)

6x loading buffer: 0.25% bromophenol blue and 20% glycerol in TAE

Appendix 3

CORRELATION ANALYSIS BETWEEN THE POSTHARVEST LESIONS AND MICROORGANISMS ISOLATED FROM THEM, AS WELL AS, THE CORRELATION BETWEEN ASYMPTOMATIC AREAS AND THE MICROORGANISMS ISOLATED THEREFROM

Recovery of bacteria and fungi from postharvest lesions

	C1	C2	C3	C4	C5	C6	C7	C8
	isol.	pseud.	bacteria	Pen.	yeast	Trich.	Chrom.	Vert.
1	72	66	4	32	4	4	0	0
2	30	28	10	0	0	0	2	0
3	54	52	2	2	0	2	0	0
4	146	146	42	22	48	0	0	0
5	70	62	11	8	0	8	2	0
6	70	39	31	14	0	0	0	0
7	70	60	54	12	0	0	0	2

```
Worksheet size: 3500 cells

MTB > Regress 'isol.' 1 'pseud.'.

The regression equation is
isol. = 15.5 + 0.891 pseud.

Predictor       Coef       Stdev    t-ratio        p
Constant      15.460       8.378       1.85    0.124
pseud.        0.8914      0.1135       7.85    0.001

S = 10.66       R-sq = 92.5%     R-sq(adj) = 91.0%

Analysis of Variance

SOURCE        DF          SS          MS          F        p
Regression     1      6999.1      6999.1      61.64    0.001
Error          5       567.7       113.5
Total          6      7566.9

Unusual Observations
Obs.   pseud.      isol.        Fit  Stdev.Fit   Residual    St.Resid
  4      146     146.00     145.60      10.07       0.40       0.12 X
  6       39      70.00      50.22       4.97      19.78       2.10R

R denotes an obs. with a large st. resid.
X denotes an obs. whose X value gives it large influence.

MTB > Regress 'isol.' 1 'bacteria'.

The regression equation is
isol. = 53.0 + 0.916 bacteria

Predictor       Coef       Stdev    t-ratio        p
Constant       53.00       19.21       2.76    0.040
bacteria      0.9158      0.6626       1.38    0.226

S = 33.09       R-sq = 27.6%     R-sq(adj) = 13.2%

Analysis of Variance

SOURCE        DF          SS          MS          F        p
Regression     1        2092        2092       1.91    0.226
Error          5        5475        1095
Total          6        7567



MTB > Regress 'isol.' 1 'Pen.'.

The regression equation is
isol. = 49.1 + 1.87 Pen.

Predictor       Coef       Stdev    t-ratio        p
Constant      49.06       18.81       2.61    0.048
Pen.          1.873       1.137       1.65    0.160
```

= 31.32 R-sq = 35.2% R-sq(adj) = 22.2%

nalysis of Variance

OURCE	DF	SS	MS	F	p
egression	1	2661.4	2661.4	2.71	0.160
rror	5	4905.4	981.1		
otal	6	7566.9			

nusual Observations

bs.	Pen.	isol.	Fit	Stdev.Fit	Residual	St.Resid
4	22.0	146.0	90.3	15.8	55.7	2.06R

denotes an obs. with a large st. resid.

TB > Regress 'isol.' 1 'yeast'.

he regression equation is
sol. = 59.7 + 1.81 yeast

redictor	Coef	Stdev	t-ratio	p
onstant	59.726	6.569	9.09	0.000
east	1.8061	0.3608	5.01	0.004

= 15.87 R-sq = 83.4% R-sq(adj) = 80.0%

nalysis of Variance

OURCE	DF	SS	MS	F	p
egression	1	6308.1	6308.1	25.06	0.004
rror	5	1258.8	251.8		
otal	6	7566.9			

nusual Observations

bs.	yeast	isol.	Fit	Stdev.Fit	Residual	St.Resid
2	0.0	30.00	59.73	6.57	-29.73	-2.06R
4	48.0	146.00	146.42	15.82	-0.42	-0.34 X

denotes an obs. with a large st. resid.
X denotes an obs. whose X value gives it large influence.

TB > Regress 'isol.' 1 'Trich.'.

he regression equation is
sol. = 75.6 - 1.21 Trich.

redictor	Coef	Stdev	t-ratio	p
onstant	75.57	17.91	4.22	0.008
rich.	-1.214	5.170	-0.23	0.824

= 38.69 R-sq = 1.1% R-sq(adj) = 0.0%

nalysis of Variance

OURCE	DF	SS	MS	F	p

```
Regression     1          83          83      0.06     0.824
Error          5        7484        1497
Total          6        7567

Unusual Observations
Obs.    Trich.      isol.        Fit  Stdev.Fit   Residual     St.Resid
  4      0.00      146.0       75.6       17.9       70.4        2.05R

R denotes an obs. with a large st. resid.

MTB > Regress 'isol.' 1 'Chrom.'.

The regression equation is
isol. = 82.4 - 16.2 Chrom.

Predictor       Coef       Stdev    t-ratio        p
Constant       82.40       15.58       5.29    0.003
Chrom.        -16.20       14.57      -1.11    0.317

s = 34.83       R-sq = 19.8%     R-sq(adj) = 3.8%

Analysis of Variance

SOURCE        DF          SS          MS         F        p
Regression     1        1500        1500      1.24    0.317
Error          5        6067        1213
Total          6        7567

Unusual Observations
Obs.    Chrom.      isol.        Fit  Stdev.Fit   Residual     St.Resid
  4      0.00      146.0       82.4       15.6       63.6        2.04R

R denotes an obs. with a large st. resid.

MTB > Regress 'isol.' 1 'Vert.'.

The regression equation is
isol. = 73.7 - 1.8 Vert.

Predictor       Coef       Stdev    t-ratio        p
Constant       73.67       15.87       4.64    0.006
Vert.          -1.83       20.99      -0.09    0.934

s = 38.87       R-sq = 0.2%      R-sq(adj) = 0.0%

Analysis of Variance

SOURCE        DF          SS          MS         F        p
Regression     1          12          12      0.01    0.934
Error          5        7555        1511
Total          6        7567

Unusual Observations
Obs.     Vert.      isol.        Fit  Stdev.Fit   Residual     St.Resid
  4      0.00      146.0       73.7       15.9       72.3        2.04R
  7      2.00       70.0       70.0       38.9        0.0            * X
```

denotes an obs. with a large st. resid,
denotes an obs. whose X value gives it large influence.

TB >

Recovery of bacteria and fungi from asymptomatic areas

	C1	C2	C3	C4	C5	C6	C7	C8
	isol.	pseud.	bacteria	Pen.	yeast	Trich.	Chrom.	Vert.
1	10	1	1	0	0	1	0	0
2	10	2	0	0	0	0	0	0
3	14	10	2	0	0	0	1	0

```
Worksheet size: 3500 cells

MTB > Correlation 'isol.' 'pseud.'.

Correlation of isol. and pseud. = 0.995

MTB > Correlation 'isol.' 'bacteria'.

Correlation of isol. and bacteria = 0.866

MTB > Correlation 'isol.' 'Pen.'.

* NOTE  * All values in column are identical.

* ERROR * Completion of computation impossible.

MTB > Correlation 'isol.' 'yeast'.

* NOTE  * All values in column are identical.

* ERROR * Completion of computation impossible.

MTB > Correlation 'isol.' 'Trich.'.

Correlation of isol. and Trich. = -0.500

MTB > Correlation 'isol.' 'Chrom.'.

Correlation of isol. and Chrom. = 1.000

MTB > Correlation 'isol.' 'Vert.'.

* NOTE  * All values in column are identical.

* ERROR * Completion of computation impossible.

MTB > Regress 'isol.' 1 'pseud.'.

The regression equation is
isol. = 9.32 + 0.466 pseud.

Predictor       Coef       Stdev    t-ratio        p
Constant      9.3151      0.2807      33.18    0.019
pseud.       0.46575     0.04745       9.81    0.065

s = 0.3310      R-sq = 99.0%     R-sq(adj) = 97.9%

Analysis of Variance

SOURCE        DF          SS          MS         F        p
Regression     1      10.557      10.557     96.33    0.065
Error          1       0.110       0.110
Total          2      10.667

Unusual Observations
Obs.   pseud.      isol.        Fit  Stdev.Fit   Residual    St.Resid
```

```
 3      10.0      14.000      13.973       0.330       0.027        1.00 X

X denotes an obs. whose X value gives it large influence.

MTB > Regress 'isol.' 1 'bacteria'.

The regression equation is
isol. = 9.33 + 2.00 bacteria

Predictor       Coef       Stdev    t-ratio         p
Constant       9.333       1.491       6.26     0.101
bacteria       2.000       1.155       1.73     0.333

s = 1.633       R-sq = 75.0%     R-sq(adj) = 50.0%

Analysis of Variance

SOURCE       DF          SS          MS          F         p
Regression    1       8.000       8.000       3.00     0.333
Error         1       2.667       2.667
Total         2      10.667


MTB > Regress 'isol.' 1 'Pen.'.
*     Pen. has all values = 0
*     Pen. has been removed from the equation

* ERROR * All variables eliminated from equation


* NOTE  * All values in column are identical.

MTB > Regress 'isol.' 1 'yeast'.
*    yeast has all values = 0
*    yeast has been removed from the equation

* ERROR * All variables eliminated from equation


* NOTE  * All values in column are identical.

MTB > Regress 'isol.' 1 'Trich.'.

The regression equation is
isol. = 12.0 - 2.00 Trich.

Predictor       Coef       Stdev    t-ratio         p
Constant      12.000       2.000       6.00     0.105
Trich.        -2.000       3.464      -0.58     0.667

s = 2.828       R-sq = 25.0%     R-sq(adj) = 0.0%

Analysis of Variance

SOURCE       DF          SS          MS          F         p
```

```
Regression     1       2.667       2.667      0.33     0.667
Error          1       8.000       8.000
Total          2      10.667

Unusual Observations
Obs.    Trich.      isol.        Fit  Stdev.Fit   Residual     St.Resid
  1      1.00      10.00      10.00       2.83       0.00           * X

X denotes an obs. whose X value gives it large influence.

MTB > Regress 'isol.' 1 'Chrom.'.

The regression equation is
isol. = 10.0 + 4.00 Chrom.

Predictor       Coef       Stdev    t-ratio         p
Constant     10.0000      0.0000          *         *
Chrom.       4.00000     0.00000          *         *

s = 0           R-sq = 100.0%    R-sq(adj) = 100.0%

Analysis of Variance

SOURCE       DF          SS          MS         F         p
Regression    1      10.667      10.667         *         *
Error         1       0.000       0.000
Total         2      10.667

Unusual Observations
Obs.    Chrom.      isol.        Fit  Stdev.Fit   Residual     St.Resid
  3      1.00    14.0000    14.0000     0.0000     0.0000           * X

X denotes an obs. whose X value gives it large influence.

MTB > Regress 'isol.' 1 'Vert.'.
*    Vert. has all values = 0
*    Vert. has been removed from the equation

* ERROR * All variables eliminated from equation


* NOTE  * All values in column are identical.

MTB >
```

Appendix 4

BACTERIA AND FUNGI RECOVERED FROM SYMPTOMATIC AREAS ON THE *AGARICUS BISPORUS* PILEI WHICH HAD THE POSTHARVEST SPOTTING DISEASE

Appendix 4. The number of isolations performed and bacteria and fungi recovered from specific symptomatic areas on the *Agaricus bisporus* pilei, which had the unusual postharvest spotting disease.

Isolations	Description Of Area: Non-spotted	Grey	Light brown	Grey brown	Dark brown	Brown	Golden brown
Total*	34	1	4	48	11	31	117
*Pseudo*monas spp.	13	0	0	6	6	8	50
Bacteria	3	1	1	9	2	3	11
*Penicill*ium spp.	0	0	1	0	0	19	4
Yeast spp.	0	0	0	3	0	2	2
Trichoderma spp.	1	0	0	4	0	0	0
Chromelosporium spp	1	0	1	0	0	1	0
*Aspergill*us spp.	0	0	0	2	0	0	0
*Verticill*ium spp.	0	0	0	0	0	1	0
	18	1	3	24	8	34	67
	53	**100**	**75**	**50**	**73**	**110**	**57**

- * = total number of isolations made from the areas described
- underlined = total number of isolations resulting in bacterial or fungal recovery
- **bold** = percentage of isolations from a particular area resulting in recovery of bacteria or fungi
- note: can get greater than 100% recovery since more than one organism may be recovered from a tissue block

Appendix 5

SIMPLE CORRELATION ANALYSIS ON THE RELATIONSHIP BETWEEN PATHOGENICITY TO MUSHROOMS AND WHITE LINE REACTIVITY

Pathogenic properties of strains of fluorescent pseudomonads

	C1	C2	C3	C4	C5
	strains	MRPT +	14340	33618	WLR -
1	60	11	11	0	49
2	38	11	11	0	27
3	50	8	8	0	42
4	73	12	12	0	61
5	57	38	38	0	19
6	58	41	43	0	15
7	59	46	46	0	13

```
Worksheet size: 3500 cells

MTB > Correlation 'MRPT +' '14340'.

Correlation of MRPT + and 14340 = 0.999

MTB > Correlation 'MRPT +' 'WLR -'.

Correlation of MRPT + and WLR - = -0.825

MTB > Correlation '14340' 'WLR -'.

Correlation of 14340 and WLR - = -0.826

MTB >
```

Appendix 6

PATHOGENICITY TRIAL RESULTS, FOR RANDOMLY SELECTED BACTERIA AND FUNGI, WHICH WERE ISOLATED FROM THE POSTHARVEST LESIONS, FOR THREE BREAKS OF THE MUSHROOM CROP

Appendix 6-a. Number of mushrooms and day of symptom development for mushrooms inoculated with randomly selected bacteria and fungi which were isolated from the postharvest lesions, for break one of the mushroom crop.

Isolate	# mushrooms inoculated	Days After Harvest:							
		1	2	3	4	5	6	7	Total
L27-10-1	150	0	0	0	0	0	0	0	**0**
L27-2-1	150	0	0	0	0	0	0	0	**0**
LII-7-1	150	0	0	0	0	0	0	0	**0**
L12-1-1	150	0	0	0	0	0	0	0	**0**
L1598-5-1	150	0	0	0	0	0	0	0	**0**
LII-2-1	150	0	0	0	0	0	0	0	**0**
L27-8-1	150	0	0	0	0	0	0	0	**0**
L1597-1-1	150	0	0	0	0	0	0	0	**0**
L27-3-1	150	0	0	0	0	0	0	0	**0**
L12-8-1	150	0	0	0	0	0	0	0	**0**
L27-12-1	150	0	0	0	0	0	0	0	**0**
Control 1	30	0	0	0	0	0	0	0	**0**
Control 2	30	0	0	0	0	0	0	0	**0**
Control 3	30	0	0	0	0	0	0	0	**0**
Non-inoc.	30	0	0	0	0	0	0	0	**0**

- mushrooms inoculated three days prior to harvest
- mushrooms stored at an average temperature of 8°C
- data represents only those mushrooms which developed the postharvest symptom of interest
- Control 1 = Luria Bertani media; Control 2 = sterile dH_2O; Control 3 = Malt Extract broth; Non-inoc = non-inoculated.

Appendix 6-b. Number of mushrooms and day of symptom development for mushrooms inoculated with randomly selected bacteria and fungi which were isolated from the postharvest lesions, for break two of the mushroom crop.

Isolate	# mushrooms inoculated	**Days** 1	**After** 2	**Harvest:** 3	4	5	6	7	Total
L27-10-1	150	0	0	0	0	0	0	0	**0**
L27-2-1	150	0	0	0	0	0	0	0	**0**
LII-7-1	150	0	0	0	0	0	0	0	**0**
L12-1-1	150	0	0	0	0	0	0	0	**0**
L1598-5-1	150	0	0	0	0	0	0	0	**0**
LII-2-1	150	0	0	0	9	11	8	2	**30**
L27-8-1	150	0	0	0	5	7	10	4	**26**
L1597-1-1	150	0	0	0	7	13	25	0	**45**
L27-3-1	150	0	6	22	0	23	5	0	**56**
L12-8-1	150	0	0	0	0	15	5	0	**20**
L27-12-1	150	0	0	0	8	9	9	9	**35**
Control 1	30	0	0	0	0	0	0	0	**0**
Control 2	30	0	0	0	0	0	0	0	**0**
Control 3	30	0	0	0	0	0	0	0	**0**
Non-inoc.	30	0	0	0	0	0	0	0	**0**
51314	50	0	0	0	0	0	0	0	**0**
51309	50	0	0	0	0	0	0	0	**0**
33618	50	0	0	0	0	0	0	0	**0**

- mushrooms inoculated three days prior to harvest
- mushrooms stored at an average temperature of 8°C
- data represents only those mushrooms which developed the postharvest symptom of interest
- Control 1 = Luria Bertani media; Control 2 = sterile dH_2O; Control 3 = Malt Extract broth; Non-inoc = non-inoculated.
- 51314 = *P. 'reactans'* ATCC 51314; 51309 = *P. tolaasii* ATCC 51309; 33618 = *P. tolaasii* ATCC 33618

Appendix 6-C. Number of mushrooms and day of symptom development for mushrooms inoculated with randomly selected bacteria and fungi which were isolated from the postharvest lesions, for break three of the mushroom crop.

Isolate	# mushrooms inoculated	Days After Harvest:							
		1	2	3	4	5	6	7	Total
L27-10-1	150	0	0	0	0	0	0	0	**0**
L27-2-1	150	0	0	0	0	0	0	0	**0**
LII-7-1	150	0	0	0	0	0	0	0	**0**
L12-1-1	150	0	0	0	0	0	0	0	**0**
L1598-5-1	150	0	0	0	0	0	0	0	**0**
LII-2-1	150	0	0	0	0	0	0	0	**0**
L27-8-1	150	0	0	0	0	0	0	0	**0**
L1597-1-1	150	0	0	0	0	0	0	0	**0**
L27-3-1	150	0	0	0	0	0	0	0	**0**
L12-8-1	150	0	0	0	0	11	0	0	**11**
L27-12-1	150	0	0	0	1	3	7	0	**11**
Control 1	30	0	0	0	0	0	0	0	**0**
Control 2	30	0	0	0	0	0	0	0	**0**
Control 3	30	0	0	0	0	0	0	0	**0**
Non-inoc.	30	0	0	0	0	0	0	0	**0**

- mushrooms inoculated three days prior to harvest
- mushrooms stored at an average temperature of 8°C
- data represents only those mushrooms which developed the postharvest symptom of interest
- Control 1 = Luria Bertani media; Control 2 = sterile dH_2O; Control 3 = Malt Extract broth; Non-inoc = non-inoculated.

Appendix 7

CHI-SQUARE ANALYSIS, TESTING THE HYPOTHESIS THAT DISEASE SYMPTOM AND ORGANISM ARE INDEPENDENT FOR THE FIRST LARGE-SCALE PATHOGENICITY TRIAL

Contingency table for the results of the large-scale pathogenicity trial

	C1	C2	C3	C4	C5	C6	C7	C8
	pen.	trich	yeast	P.put.	p.sp.	LII-2-1	L27-8-1	1597
1	0	0	0	0	0	3.94989	5.25104	9.18353
2	330	330	330	330	330	5.15070	1.61779	3.00799

Contingency table for the results of the large-scale pathogenicity trial

	C9	C10	C11
	L27-3-1	L12-8-1	L27-12-1
1	5.06146	3.5838	10.1957
2	4.13238	10.3743	1.2813

```
Worksheet size: 3500 cells

MTB > ChiSquare 'LII-2-1'-'L27-12-1'.

Expected counts are printed below observed counts

       LII-2-1  L27-8-1      1597  L27-3-1  L12-8-1 L27-12-1     Total
  1         30       26        45       56       31       46       234
       39.00    39.00    39.00    39.00    39.00    39.00

  2        300      304       285      274      299      284      1746
      291.00   291.00   291.00   291.00   291.00   291.00

Total      330      330       330      330      330      330      1980

ChiSq =  2.077 +  4.333 +  0.923 +  7.410 +  1.641 +  1.256 +
         0.278 +  0.581 +  0.124 +  0.993 +  0.220 +  0.168 = 20.005
df = 5

MTB > Let K2=1-K1
MTB > Let K2=1-K1
                S
*** Empty column, undefined or illegal stored constant at S


* ERROR * Completion of computation impossible.

MTB > Let K2 = 1 - K1
MTB > Let K2 = 1 - K1
                  S
*** Empty column, undefined or illegal stored constant at S


* ERROR * Completion of computation impossible.

MTB > PDF 20.005 K2;
SUBC>   Chisquare 5.
MTB > Let K2=1-K1
MTB > Let K2=1-K1
                S
*** Empty column, undefined or illegal stored constant at S


* ERROR * Completion of computation impossible.

MTB > Random 2 'LII-2-1'-'L27-12-1';
SUBC>   Chisquare 5.
MTB > Let K2=1-K1
MTB > Let K2=1-K1
                S
*** Empty column, undefined or illegal stored constant at S


* ERROR * Completion of computation impossible.

MTB > CDF 20.005 K2;
```

```
MTB>   Chisquare 5.
MTB > Let K1=1-K2
MTB > print K1

K1          0.00124151
MTB >
```

Appendix 8

PATHOGENICITY OF *PSEUDOMONAS TOLAASII* AT DIFFERENT INOCULUM LEVELS, NUMBER OF MUSHROOMS WHICH DEVELOP POSTHARVEST SPOTTING AND TIME TO SYMPTOM DEVELOPMENT

Appendix 8. Pathogenicity of *Pseudomonas tolaasii* (57) at various inoculum levels, numbers of mushrooms which develop postharvest spotting and time to symptom development.

Concentration (cfu's)	Break	# mushrooms inoculated	Days After Harvest							
			1	2	3	4	5	6	7	Total
10^5	1	50	10	2	0	0	0	0	0	12
	2	50	23	0	5	0	0	1	0	29
	3	50	12	1	0	0	0	0	0	13
10^3	1	50	0	0	1	11	7	0	0	19
	2	50	0	0	4	29	9	0	1	43
	3	50	2	0	0	33	1	1	0	37
10^1	1	50	0	0	0	0	0	0	1	1
	2	50	0	0	0	0	0	1	4	5
	3	50	0	0	0	0	0	0	2	2

- mushrooms inoculated three days prior to harvest and stored at an average temperature of 8°C
- data for higher concentrations not shown since all mushrooms developed symptoms prior to harvest

Appendix 9

WALD'S CHI-SQUARE ANALYSIS

TABLE5

The LOGISTIC Procedure

ata Set: WORK.TABLE5
esponse Variable (Events): B1I
esponse Variable (Trials): TOTB1
umber of Observations: 5
ink Function: Logit

Response Profile

Ordered Value	Binary Outcome	Count
1	EVENT	132
2	NO EVENT	118

NING: 1 observation(s) were deleted due to missing values for the response or explanatory variables.

Model Fitting Information and Testing Global Null Hypothesis BETA=0

Criterion	Intercept Only	Intercept and Covariates	Chi-Square for Covariates
AIC	347.789	183.056	.
SC	351.311	190.099	.
-2 LOG L	345.789	179.056	166.733 with 1 DF (p=0.0001)
Score	.	.	133.547 with 1 DF (p=0.0001)

Analysis of Maximum Likelihood Estimates

ariable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square	Standardized Estimate	Odds Ratio
NTERCPT	1	-4.1358	0.5220	62.7746	0.0001	.	.
OSE	1	0.8774	0.1025	73.2471	0.0001	1.370921	2.405

Association of Predicted Probabilities and Observed Responses

Concordant = 87.9%	Somers' D = 0.828
Discordant = 5.1%	Gamma = 0.891
Tied = 7.0%	Tau-a = 0.414
(15576 pairs)	c = 0.914

TABLE5

The LOGISTIC Procedure

ta Set: WORK.TABLE5
sponse Variable (Events): B2I
sponse Variable (Trials): TOTB2
mber of Observations: 5
nk Function: Logit

Response Profile

Ordered Value	Binary Outcome	Count
1	EVENT	177
2	NO EVENT	73

NING: 1 observation(s) were deleted due to missing values for the response or explanatory variables.

Model Fitting Information and Testing Global Null Hypothesis BETA=0

Criterion	Intercept Only	Intercept and Covariates	Chi-Square for Covariates
AIC	303.966	196.582	.
SC	307.488	203.625	.
-2 LOG L	301.966	192.582	109.384 with 1 DF (p=0.0001)
Score	.	.	91.024 with 1 DF (p=0.0001)

Analysis of Maximum Likelihood Estimates

ariable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square	Standardized Estimate	Odds Ratio
NTERCPT	1	-1.9577	0.3543	30.5360	0.0001	.	.
OSE	1	0.7108	0.0927	58.7987	0.0001	1.110612	2.036

Association of Predicted Probabilities and Observed Responses

Concordant	= 83.1%	Somers' D	= 0.751
Discordant	= 8.1%	Gamma	= 0.823
Tied	= 8.8%	Tau-a	= 0.312
(12921 pairs)		c	= 0.875

TABLE5

The LOGISTIC Procedure

ata Set: WORK.TABLE5
esponse Variable (Events): B3I
esponse Variable (Trials): TOTB3
umber of Observations: 6
ink Function: Logit

Response Profile

Ordered Value	Binary Outcome	Count
1	EVENT	202
2	NO EVENT	98

Model Fitting Information and Testing Global Null Hypothesis BETA=0

Criterion	Intercept Only	Intercept and Covariates	Chi-Square for Covariates
AIC	381.076	222.864	.
SC	384.779	230.272	.
-2 LOG L	379.076	218.864	160.211 with 1 DF (p=0.0001)
Score	.	.	129.709 with 1 DF (p=0.0001)

Analysis of Maximum Likelihood Estimates

ariable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square	Standardized Estimate	Odds Ratio
NTERCPT	1	-2.5582	0.3618	50.0053	0.0001	.	.
OSE	1	0.6694	0.0763	76.9547	0.0001	1.262696	1.953

Association of Predicted Probabilities and Observed Responses

Concordant = 87.2%	Somers' D = 0.798
Discordant = 7.4%	Gamma = 0.843
Tied = 5.3%	Tau-a = 0.352
(19796 pairs)	c = 0.899

TABLE5

The LOGISTIC Procedure

ata Set: WORK.TABLE5
esponse Variable (Events): B123I
esponse Variable (Trials): TOTB123
umber of Observations: 6
ink Function: Logit

Response Profile

Ordered Value	Binary Outcome	Count
1	EVENT	511
2	NO EVENT	289

Model Fitting Information and Testing Global Null Hypothesis BETA=0

Criterion	Intercept Only	Intercept and Covariates	Chi-Square for Covariates
AIC	1048.614	629.004	.
SC	1053.299	638.373	.
-2 LOG L	1046.614	625.004	421.610 with 1 DF (p=0.0001)
Score	.	.	341.550 with 1 DF (p=0.0001)

Analysis of Maximum Likelihood Estimates

ariable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Square	Standardized Estimate	Odds Ratio
NTERCPT	1	-2.6509	0.2192	146.2742	0.0001	.	.
OSE	1	0.7021	0.0480	214.1782	0.0001	1.200733	2.018

Association of Predicted Probabilities and Observed Responses

Concordant = 85.1%	Somers' D = 0.778
Discordant = 7.2%	Gamma = 0.843
Tied = 7.7%	Tau-a = 0.360
(147679 pairs)	c = 0.889

Appendix 10

SCANNING DENSITOMETRIC ANALYSIS OF ELECTROPHORETIC PROTEIN PATTERNS OF REPRESENTATIVE MEMBERS OF *PSEUDOMONAS TOLAASII* STRAINS

Scanning densitometric analysis of SDS-PAGE 1

	C1	C2	C3	C4	C5	C6
	51314	51309	33618	57-1	57-2	57-3
1	42.088	23.541	42.099	33.088	37.480	37.768
2	29.457	16.870	40.352	38.997	46.044	43.787
3	90.959	75.090	127.933	78.297	71.565	48.551
4	84.835	72.348	125.044	120.776	92.461	71.608
5	30.510	27.117	59.160	95.477	82.787	59.255
6	19.902	31.291	68.497	106.504	111.047	82.903
7	1.861	17.170	56.027	70.811	77.292	70.849
8	0.129	9.051	40.152	81.274	79.616	

rksheet size: 3500 cells

TB > Correlation '51314'-'57-3'.

	51314	51309	33618	57-1	57-2
1309	0.940				
3618	0.864	0.982			
7-1	0.226	0.484	0.585		
7-2	-0.042	0.263	0.390	0.929	
7-3	-0.359	-0.054	0.099	0.773	0.898

TB >

Appendix 11

SCANNING DENSITOMETRIC ANALYSIS OF ELECTROPHORETIC PROTEIN PATTERNS OF *PSEUDOMONAS TOLAASII* STRAINS WHICH WERE ISOLATED FROM THE POSTHARVEST LESIONS

Scanning densitometric analysis of gel 2

	C1	C2	C3	C4	C5	C6	C7
	57-1	type	blotch-1	57-2	blotch-2	57-3	blotch-3
1	65.102	60.988	6.742	29.795	2.095	31.553	9.283
2	57.820	91.765	32.158	59.920	9.451	34.303	17.901
3	107.043	106.195	183.959	191.196	125.465	94.306	185.985
4	112.313	121.752	190.785	149.548	153.481	95.255	130.574
5	84.415	78.194	154.831	97.771	65.230	70.655	82.493
6	144.166	87.793	264.831	147.145	172.330	120.026	196.098

```
orksheet size: 3500 cells

TB > Correlation 'type'-'blotch-3'.

           type blotch-1     57-2 blotch-2     57-3
lotch-1  0.525
7-2      0.750    0.860
lotch-2  0.658    0.960    0.889
7-3      0.555    0.990    0.885    0.984
lotch-3  0.561    0.943    0.944    0.943    0.968

TB > Correlation 'type'-'blotch-3'.

           type blotch-1     57-2 blotch-2     57-3
lotch-1  0.525
7-2      0.750    0.860
lotch-2  0.658    0.960    0.889
7-3      0.555    0.990    0.885    0.984
lotch-3  0.561    0.943    0.944    0.943    0.968

TB > Retrieve  'C:\MTBSEW\STUDENT9\SDSPAGE.MTW'.
etrieving worksheet from file: C:\MTBSEW\STUDENT9\SDSPAGE.MTW
orksheet was saved on  7/ 2/2000
TB > Correlation '57-1'-'b-3'.

           57-1        T      b-1     57-2      b-2     57-3
          0.678
-1        0.820    0.525
7-2      0.975    0.763    0.846
-2        0.811    0.488    0.997    0.829
7-3      0.983    0.751    0.815    0.997    0.799
-3        0.811    0.478    0.998    0.827    0.999    0.797

TB >
```